Lactobacillus reuteri glucosyltransferase

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(54) Title: LACTOBACILLUS REUTERI GLUCOSYLTRANSFERASE

(57) Abstract: The present invention describes a protein having glucosyltransferase activity. This protein is derived from lactobacilli, which are food-grade microorganisms with the Generally Recognised As Safe (GRAS) status. The protein produces a glucan with a unique structure having 4-linked, 6-linked and 4,6-linked anhydroglucose units or in the presence of suitable acceptors, oligosaccharides. According to the invention lactobacilli capable of producing this glucan using the novel glucosyltransferase can be used as a probiotic or symbiotic.

LACTOBACILLUS REUTERI GLUCOSYLTRANSFERASE

[0001] The present invention is in the field of enzymatic production of biomolecules. The invention is particularly concerned with a novel type of glucosyltransferase derived from lactobacilli and with a process for recombinant production of the enzyme and for the production of useful glucans and gluco-oligosaccharides from sucrose.

Background of the invention

[0002] Lactic acid bacteria (LAB) play an important role in the fermentative production of food and feed. Traditionally, these bacteria have been used for the production of for instance wine, beer, bread, cheese and yoghurt, and for the preservation of food and feed, e.g. olives, pickles, sausages, sauerkraut and silage. Because of these traditional applications, lactic acid bacteria are food-grade micro-organisms that posses the Generally Recognised As Safe (GRAS) status. Due to the different products which are formed during fermentation with lactic acid bacteria, these bacteria contribute positively to the taste, smell and preservation of the final product. The group of lactic acid bacteria includes several genera such as *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, etc.

[0003] In recent years also the health promoting properties of lactic acid bacteria have received much attention. They produce an abundant variety of exopolysaccharides (EPS's). These polysaccharides are thought to contribute to human health by acting as prebiotic substrates, nutraceuticals, cholesterol lowering agents or immunomodulants.

[0004] To date high molecular weight polysaccharides produced by plants (such as cellulose, starch and pectin), seaweeds (such as alginate and carrageenan) and bacteria (such as alginate, gellan and xanthan) are used in several industrial applications as viscosifying, stabilising, emulsifying, gelling or water binding agents. Although all these polysaccharides are used as food additives, they originate from organisms not having the GRAS status. Thus they are less desirable than the exopolysaccharides of micro-organisms, such as lactic acid bacteria, which have the GRAS status.

[0005] The exopolysaccharides produced by lactic acid bacteria can be divided in two groups, heteropolysaccharides and homopolysaccharides; these are synthesised by totally different mechanisms. The former consist of repeating units in which residues of different types of sugars are present and the latter consist of one type of monosaccharide. The synthesis of heteropolysaccharides by lactic acid bacteria, including lactobacilli, has been studied extensively in recent years. Considerable less information is present on the synthesis of homopolysaccharides from lactobacilli, although some studies have been

performed. The information on the synthesis of homopolysaccharides in lactobacilli is mainly limited to the synthesis of glucans and only two reports, written by the present inventors, exist on the synthesis of fructans. In one of these reports the *Lactobacillus reuteri* strain LB 121 was found to produce both a glucan and a fructan when grown on sucrose, but only a fructan when grown on raffinose (van Geel-Schutten, G.H. *et al.*, Appl. Microbiol. Biotechnol. (1998) 50, 697-703). In the other report was found that *Lactobacillus reuteri* strain LB 35-5, a spontaneous mutant of *Lactobacillus reuteri* strain LB 121, only produced a glucan when grown on sucrose (van Geel-Schutten, G.H. *et al.*, Appl. Environ. Microbiol. (1999) 65, 3008-3014). In the other report the soluble glucan and fructan were also characterised by their molecular weights (of 3,500 and 150 kDa respectively) and the glucan was reported to be highly branched with a unique structure consisting of a terminal, 4-substituted, 6-substituted, and 4,6-di-substituted α-glucose in a molar ratio 1.1 : 2.7 : 1.5 : 1.0 (van Geel-Schutten, G.H. *et al.*, Appl. Environ. Microbiol. (1999) 65, 3008-3014). No structurally identification of a similar glucan produced by a *Lactobacillus* had been reported before. The fructan was identified as a (2→6)-β-D-fructofuranan (also called a levan). This was the first example of levan synthesis by a *Lactobacillus* species.

Summary of the invention

[0006] A novel enzyme having glucosyltransferase activity using sucrose as a substrate has now been found in *Lactobacillus reuteri*, and its amino acid sequence and other structural properties have been determined. The enzyme is unique in that it is capable of producing a highly branched glucan with α-1,4- and α-1,6-glucosidic links. The invention thus pertains to an enzyme, to DNA encoding it, to cells containing such DNA and to their use in producing carbohydrates, as defined in the appending claims. The invention also pertains to glucans so produced as well as to oligosaccharides and chemically derivatised glucans.

Description of the invention

[0007] It was found according to the invention that the glucans are produced by certain *Lactobacillus* strains, in particular by certain strains of *Lactobacillus reuteri*, as a result of the activity of a single glucosyltransferase (glucansucrase).

[0008] The nucleotide and amino acid sequence of the novel glucosyltransferase are represented by SEQ ID No. 1 and 2, respectively, and are also shown in figure 3. The start codon of the glucosyltransferase is preceded by a putative ribosome binding site with the

nucleotide sequence GAAGGAGA (located 5 base pairs upstream of the start codon of the glucosyltransferase; see SEQ ID No. 1 and figure 3). Furthermore, the start codon is preceded by the nucleotide sequence TATAAT, also called Pribnow box or -10 region, (located 42 base pairs upstream of the start codon; see SEQ ID No. 1 and figure 3) and by the nucleotide sequence TTGAAA, also called -35 region (located 80 base pairs upstream of the start codon; see SEQ ID No. 1 and figure 3).

[0009] The invention covers a protein having glucosyltransferase activity with sucrose as substrate comprising an amino acid sequence with an amino acid identity of at least 50%, preferably at least 60%, and more preferably at least 70%, compared to the amino acid sequence 531-1781 of SEQ ID No. 2. The invention also covers a part of a protein with at least 15 contiguous amino acids which are identical to the corresponding part of the amino acid sequence 531-1781 of SEQ ID No. 2. The present invention covers a protein having glucosyltransferase activity with sucrose as donor substrate with an amino acid identity of at least 50%, preferably at least 60%, and more preferably at least 70%, compared to the amino acid sequence of SEQ ID No. 2. The invention also covers a part of a protein with at least 15 contiguous amino acids which are identical to the corresponding part of the amino acid sequence of SEQ ID No. 2. The novel glucosyltransferase has homology with several other proteins as revealed by amino acid sequence alignment. A high homology (figure 5) was found with an alternansucrase of *Leuconostoc mesenteroides* strain NRRL B-1355 (46% identity, within 1261 amino acids of amino acid sequence 531-1781 of SEQ ID No. 2) and a dextransucrase of *Leuconostoc mesenteroides* strain NRRL B-512F (44% identity, within 1270 amino acids of amino acid sequence 531-1781 of SEQ ID No. 2). Furthermore, the alignment revealed the presence of various domains also found in the other glucosyltransferases, such as an N-terminal variable domain, a catalytic domain and a C-terminal glucan binding domain. The N-terminal domain shows almost no identity with the N-terminal domains of other glucosyltransferases and an N-terminal signal peptide could not be detected.

[0010] The invention also covers a protein comprising an amino acid sequence of at least 100 amino acids, exhibiting at least 55%, preferably at least 65% amino acid identity with the corresponding part of the amino acid sequence 972-1514 (catalytic domain) of SEQ ID No. 2. The catalytic domain shows a high level of homology (about 50% identity) with other known streptococcal and *Leuconostoc* glucosyltransferases and putative functions based on the alignment can be ascribed to several amino acids within this catalytic domain (figure 4). Asp-1024, Glu-1061 and Asp-1133 of SEQ ID No. 2 are putative catalytic

residues, Asp-984 of SEQ ID No. 2 is a putative calcium binding residue and Arg-1022 of SEQ ID No. 2 a putative chloride binding residue. His-1132 and Gln-1514 of SEQ ID No. 2 may stabilise the transition state and the residues Asp-1027, Asn-1028, Asp-1062 and Trp-1063 of SEQ ID No. 2 may play a role in binding of acceptor molecules and in the transfer of the glucosyl moiety.

[0011] The invention further covers a protein comprising an amino acid sequence of at least 100 amino acids, exhibiting at least 50%, preferably at least 60%, amino acid identity with the corresponding part of the amino acid sequence 1515-1781 (glucan binding domain) of SEQ ID No. 2. The C-terminal putative glucan binding domain is much shorter than the corresponding domains in other glucosyltransferases but three known repeats, resembling YG repeats, are described: YYFYDLAGNMVKN (SEQ ID No. 3) starting at amino acid position 1656 of SEQ ID No. 2, WYFFDQDGKMVEN (SEQ ID No. 4) starting at amino acid position 1678 of SEQ ID No. 2 and TYYFDNYGKMVRN (SEQ ID No. 5) starting at amino acid position 1725 of SEQ ID No. 2. YG repeats are defined by the presence of one or more aromatic residues (of which one is usually tyrosine), followed by 3-4 glycine residues downstream, a hydrophobic residue, a neutral polar residue (usually glycine or asparagine) and 1-3 hydrophobic residues. It is striking that the number of repeats necessary to ensure glucan binding properties is different for enzymes producing a soluble or an insoluble glucan. Possibly the glucan binding domain is also involved in the determination of the glucan structure and the polymer chain growth. Furthermore, this domain seems also necessary for the complete glucosyltransferase activity.

[0012] Specific amino acids of the glucosyltransferase that are believed to be important for the unique properties of the enzyme include Pro-1026, Ile-1029, Met-1034, Asn-1035, Ser-1136, Ala-1143, Ile-1170, Leu-1223, Ala-1413, Val-1418, Ala-1428, Leu-1442 of the amino acid sequence of SEQ ID No. 2. So a protein, mutant or part thereof, comprising at least one of the above mentioned amino acids is also part of the invention. Particularly Pro-1026 and Ile-1029 are of interest. Pro-1026 is found in a position where a conserved Val is found in other glucosyltransferases. Compared to Val, the presence of Pro results in a more rigid protein structure. This change of protein structure may influence the glucosidic bonds formed and might explain the unique structure of the glucan. Ile-1029 is also found in a position where a conserved Val is present in other LAB glucosyltransferases not producing $\alpha(1,4)$ bonds. An identical amino acid substitution is observed in amylosucrase, a glucosyltransferase synthesising $\alpha(1,4)$ bonds.

[0013] A nucleotide sequence encoding any of the above mentioned proteins, mutants, variants or parts thereof is also a subject of the invention. Furthermore, the nucleic acid sequences corresponding to expression-regulating regions (promoters, enhancers, terminators) contained in the nucleic acid sequence (1)-(160) or (5507)-(6026) of SEQ ID No. 1 (see also figure 3) can be used for homologous or heterologous expression of genes. Such expression-regulating sequences are operationally linked to a polypeptide-encoding nucleic acid sequence such as the genes of the glucosyltransferase according to the invention. Inverted repeats are located 62 base pairs downstream the termination codon (TAA), suggesting the presence of a Rho independent transcription termination signal. The -10 and -35 consensus promoter sequences, two motifs generally present upstream of the start codon of procaryotes, are identified as described above. Other promoter, enhancer or terminator were not identified. A nucleic acid construct comprising the nucleotide sequence operationally linked to an expression-regulating nucleic acid sequence is also covered by the invention.

[0014] A recombinant host cell, such as a mammalian (with the exception of human), plant, animal, fungal or bacterial cell, containing one or more copies of the nucleic acid construct mentioned above is an additional subject of the invention. A part of the gene encoding the N-terminal part, the catalytic domain and the glucan-binding domain of the glucosyltransferase has been cloned and expressed in *E. coli*. The molecular weight predicted from the deduced amino acid sequence of that part (*i.e.* the amino acid sequence 531-1781 of SEQ ID No. 2; see examples) of the recombinant glucansucrase expressed in *E. coli* is 145 kDa.

[0015] The invention further covers a enzymatically active protein as defined above which, in the presence of sucrose, produces a glucan having 38-48% 4-linked anhydro-glucose units, 17-28% 6-linked anhydroglucose units, and 7-20% 4,6-linked anhydro-glucose units, preferably a glucan having 40-46% 4-linked anhydroglucose units, 19-26% 6-linked anhydroglucose units, and 9-18% 4,6-linked anhydroglucose units. There is a large variation in glucans due to differences in the type of bonds present, degree and type of branching, length of the glucan chains, molecular weight, and the conformation of the polymers. The structure of this glucan is unique in that it is highly branched, consists of terminal, 4-substituted, 6-substituted, and 4,6-di-substituted α-glucose in a molar ratio 1.1 : 2.7 : 1.5 : 1.0 and has a high molecular weight of 3500 kDa. The novel glucan may be synthesised by a glucosyltransferase present in the *Lactobacillus* strains, preferably *Lactobacillus reuteri* strains and more preferably *Lactobacillus reuteri* strains LB 121 and

LB 35-5. *Lactobacillus reuteri* belongs to the group of lactic acid bacteria which are known to play an important role in the fermentative production of food and feed. Because of this, lactic acid bacteria are food-grade micro-organisms that posses the Generally Recognised As Safe (GRAS) status.

[0016] The invention also pertains to a process of producing a glucan as described above using an isolated glucosyltransferase according to the invention and a suitable glucose source such as for instance sucrose. The glucosyltransferase may be isolated by conventional means from the culture of a glucosyltransferase-positive lactic acid bacterium, especially a *Lactobacillus reuteri*, or from a recombinant organism expressing the glucosyltransferase gene.

[0017] Additionally, the invention concerns a process of producing gluco-oligosaccharides containing the characteristic structure of the glucan described above using an isolated glucosyltransferase according to the invention. There is a growing interest in oligosaccharides derived from homopolysaccharides, for instance for prebiotic purposes. Several fructo- and gluco-oligosaccharides are known to stimulate the growth of bifidobacteria in the human colon. Gluco-oligosaccharides produced by the glucosyltransferase described above can be used as prebiotics and probiotics and are also part of the invention. The production of the gluco-oligosaccharides is different from the glucan synthesis reaction. In addition to sucrose, the substrate of the glucosyltransferase, an acceptor molecule such as maltose or lactose is necessary for the acceptor reaction. Another way of producing gluco-oligosaccharides is by hydrolysis of the glucan described above. This hydrolysis can be performed by known hydrolysis methods such as enzymatic hydrolysis with enzymes such as amylase, dextranase or pullulanase or by acid hydrolysis. The produced gluco-oligosaccharides must contain at least one 1,6- or one 4,6- glucosidic link to be used as prebiotics.

[0018] The invention also concerns chemically modified glucans and gluco-oligosaccharides based on the 1,4/1,6-α-glucans described above. Chemical modification can be achieved by oxidation, such as hypochlorite oxidation resulting in ring-opened 2,3-dicarboxy-anhydroglucose units (see e.g. EP-A-427349), periodate oxidation resulting in ring-opened 2,3-dialdehyde-anhydroglucose units (see e.g. WO 95/12619), which can be further oxidised to (partly) carboxylated units (see e.g. WO 00/26257), TEMPO-mediated oxidation resulting in 6-carboxy-anhydroglucose units (see e.g. WO 95/07303). The oxidised glucans have improved water-solubility, altered viscosity and a retarded fermentability and can be used as metal-complexing agents, detergent additives,

strengthening additives, bioactive carbohydrates, emulsifiers and water binding agents. They can also be used as starting materials for further derivatisation such as cross-linking and the introduction of hydrophobes. Oxidised glucans coupled to proteins can be used as emulsifiers and stabilisers. (Partial) hydrolysis of glucans according to the invention and/or modified glucans results in gluco-oligosaccharides, which can be used as bioactive carbohydrates or prebiotics. The oxidised glucans of the invention preferably contain 0.05-1.0 carboxyl groups per anhydroglucose unit, *e.g.* as 6-carboxyl units.

[0019] Another type of chemical modification is phosphorylation, as described in O.B. Wurzburg (1986), Modified Starches: properties and uses. CRC Press Inc., Boca Raton, 97-112. One way to achieve this modification is by dry heating glucans with a mixture of monosodium and disodium hydrogen phosphate or with tripolyphosphate. The phosphorylated glucans are suitable as wet-end additives in papermaking, as binders in paper coating compositions, as warp sizing-agents, and as core binders for sand molds for metal casting. A further type of derivatisation of the glucans is acylation, especially acetylation using acetic or propionic anhydride, resulting in products suitable as bleaching assistants and for the use in foils. Acylation with e.g. alkenyl succinic anhydrides or (activated) fatty acids results in surface-active products suitable as e.g. surfactants, emulsifiers, and stabilisers.

[0020] Hydroxyalkylation, carboxymethylation, and amino-alkylation are other methods of chemical derivatisation of the glucans. Hydroxyalkylation is commonly performed by base-catalysed reaction with alkylene oxides, such as ethylene oxide, propylene oxide or epichlorohydrine; the hydroxyalkylated products have improved solubility and viscosity characteristics. Carboxymethylation is achieved by reaction of the glucans with mono-chloroacetic acid or its alkali metal salts and results in anionic polymers suitable for various purposes including crystallisation inhibitors, and metal complexants. Amino-alkylation can be achieved by reaction of the glucans with alkylene-imines, halo-alkyl amines or amino-alkylene oxides, or by reaction of epichlorohydrine adducts of the glucans with suitable amines. These products can be used as cationic polymers in a variety of applications, especially as a wet-end additive in paper making to increase strength, for filler and fines retention, and to improve the drainage rate of paper pulp. Other potential applications include textile sizing and wastewater purification. The above mentioned modifications can be used either separately or in combination depending on the desired product. Furthermore, the degree of chemical modification is variable and depends on the intended use. If necessary 100% modification, i.e. modification of all anhydroglucose

units can be performed. However, partial modification, e.g. from 1 modified anhydroglucose unit per 100 up to higher levels, will often be sufficient in order to obtain the desired effect.

[0021] The use of a *Lactobacillus* strain capable of producing the novel and unique glucan is also covered by the invention. Preferably, the strain is also capable of producing a fructan, which can be either a levan, inulin or both. More preferably, the strain is also capable of producing fructo-oligosaccharides. For producing this glucan and/or fructan the strain according to the invention preferably uses sucrose as a substrate. The glucan according to the invention can be produced when the strain is cultured in the presence of sucrose and/or after the strain was cultured in the presence of sucrose during a sufficient time period. The *Lactobacillus* strains used have been deposited at the BCCM/LMG Culture collection (Gent, BE) under the deposit numbers LMG P-18388 (*L. reuteri* wild-type strain LB 121) and LMG P-18389 (*L. reuteri* mutant strain LB 35-5). The efficacy of some *L. reuteri* strains as a probiotic has been demonstrated in various animals such as for instance poultry and humans. The administration of *L. reuteri* to pigs resulted in significantly lower serum total and LDL-cholesterol levels, while in children *L. reuteri* is used as a therapeutic agent against acute diarrhea. For this and other reasons *L. reuteri* has already been supplemented to commercially available probiotic products. The mode of action of *L. reuteri* as a probiotic is still unclear. Preliminary studies indicated that gut colonisation by *L. reuteri* may be of importance. According to the invention, it was found that the mode of action of *L. reuteri* as a probiotic may reside partly in the ability to produce polysaccharides. *Lactobacillus* strains, preferably *L. reuteri* strains, more preferably *L. reuteri* strains LB 121, LB 35-5 and other strains capable of producing a glucan having 38-48% 4-linked anhydroglucose units (AGU), 17-28% 6-linked AGU, and 7-20% 4,6-linked AGU, preferably a glucan having 40-46% 4-linked AGU, 19-26% 6-linked AGU, and 9-18% 4,6-linked AGU can thus advantageously be used as a probiotic. They can also, together with these polysaccharides, be used as a symbiotic.

Examples

Example 1: Isolation of DNA from *Lactobacillus reuteri*, nucleotide sequence analysis of the glucosyltransferase gene, construction of plasmids for expression of the glucosyl-transferase gene in *E. coli* DH5α, expression of an essential part of the glucosyltransferase gene in *E. coli* DH5α, and identification of the novel glucan produced by the recombinant enzyme.

General procedures for cloning, DNA manipulations and agarose gel electrophoresis were essentially as described by Sambrook *et al.* (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York. Restriction endonuclease digestions and ligations with T4 DNA ligase were performed as recommended by the suppliers. DNA was amplified by PCR techniques using ampliTAQ DNA polymerase (Perkin Elmer). DNA fragments were isolated from agarose gels using the Qiagen extraction kit (Qiagen GMBH), following the instructions of the suppliers. *Lactobacillus reuteri* strains were grown anaerobically at 37°C in MRS medium (DIFCO) or in MRS-s medium (MRS medium containing 100 g/l sucrose instead of 20 g/l glucose) and *E. coli* strains were grown aerobically at 37°C in LB medium containing 100 µg/ml ampicillin (when appropriate 40 µg/ml X-gal was added).

For the isolation of chromosomal DNA, *L. reuteri* 121 was grown overnight at 37°C in MRS broth (Difco) supplemented with 40 mM DL-threonine. Cells of 4 ml culture were harvested by centrifugation and resuspended in 10 ml MRS broth supplemented with 40 mM DL-threonine and incubated for 2 h at 37°C. After centrifugation the cells were resuspended in 400 µl protoplast buffer (10 mM sodium maleate, pH 6.1 supplemented with 0.3 M lactose, 10 mM $MgCl_2$, 12% polyethyleneglycol 2000, 0.1 M EDTA, 5 mg/ml lysozyme (47,000 U/mg) and 10 U/ml mutanolysine) and incubated for 1 h at 37°C. After centrifugation (1 min, Eppendorf centrifuge), protoplasts were resuspended in 500 µl 20 mM Tris-HCl, pH 8,0. Subsequently, 100 µl laurylsarcosine and 150 µl 5 M NaCl were added and DNA was extracted. Plasmid DNA of *Lactobacillus reuteri* was isolated using a modification of the methods of Anderson and Mc Kay (1983) Appl. Environ. Microbiol. 46, 549-552 and Burger and Dicks (1994) Biotechnol. Technol. 8, 769-772. Fresh prewarmed (37°C) MRS broth (10 ml) was inoculated with 200 µl of an overnight culture and incubated for 2.5 h at 37°C. Cells were harvested by centrifugation and washed with 2 ml sterile STE buffer (0.1 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 8). After centrifugation, the pellet was resuspended in 380 µl solution I (0.5 M sucrose, 50 mM Tris-HCl, 1 mM EDTA, pH 8, containing 2 mg/ml lysozyme and 6.6 U mutanolysin). After an incubation of 1.5 h at 37°C, 50 µl of solution II (50 mM Tris-HCl, pH 80, 0,25 M EDTA) and 30 µl of solution III (50 mM Tris-HCL, pH 8, 20 mM EDTA, 20% SDS) were added and the suspension was mixed. Sodium hydroxide (30 µl of a 3 M solution) was added, followed by 50 µl 2 M Tris-HCl and 72 µl

5 N NaCl. After extraction with equal volumes of phenol and chloroform, the DNA was precipitated with ethanol.

The glucosyltransferase (*gtfA*) gene was identified by amplification with PCR using degenerated primers (GTFpr1, 5'GAYAAKWSNAAKSYNRTNGTNSARGC3' (SEQ ID No. 6) and GTFpr2, 5'GNKCNCANATRATRCCNCTRNA3' (SEQ ID No. 7); Y=T or C, K=G or T, W=A or T, S=C or G, R=A or G, N= A, C, G, or T) based on conserved amino acid sequences deduced from different glucosyltranferase genes (*gtfS* of *Streptococcus downei, gtfC* of *S. mutans, gtfI* of *S. downei, gtfK* and *gtfM* of *S. salivarius* and *dsrA* of *Leuconostoc mesenteroides*) and *Lactobacillus reuteri* chromosomal DNA as template. An amplification product with the predicted size of about 660 bp was obtained (figure 1A). To investigate the possible presence of multiple copies of the glucosyl-transferase gene, Southern hybridisation was performed. DNA was restricted with endonucleases, separated by agarose gel electrophoresis and transferred to a Hybond nylon membrane. For hybridisation, probes were labelled wih [α-^{32}P]dCTP using Random Primed DNA labelling kit (Boehringer Mannheim), following the manufacturer's instructions. The Southern hybridisation of chromosomal DNA of the *Lactobacillus reuteri* strain 121 with the amplified 660 bp PCR fragment, followed by washing under non-stringent conditions (45°C, 0.5x SSC/0.1 SDS) revealed one hybridising fragment, suggesting the presence of only a single copy of a glucosyltransferase gene in the *L. reuteri* strains. The 660 bp fragment was cloned in *E. coli* JM109 using the pCR2.1 vector. Transformations were performed by electroporation using the BioRad gene pulser apparatus at 2.5 kV, 25 μF and 200 Ω, following the instructions of the manufacturer. The fragment was sequenced by the method of Sanger *et al.* (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467, confirming that the correct part of the *gtfA* gene had been isolated. The 660 bp amplified fragment was used to design primers for inverse PCR. Using inverse PCR techniques a 3 kb fragment of the isolated *gtfA* gene was generated (figure 1B). This 3 kb amplicon was identified by sequencing and probes were designed to isolate the *Eco*RI/*Bgl*II and *Eco*RI/*Hind*III fragments from a partial DNA library of *L. reuteri* in *E. coli* DH5α (figure 1C). Positive clones were selected by colony blot hybridisation using Hybond-N filters, following the instructions of the supplier and the cloned fragments were sequenced. Attempts to clone the C-terminal part of the glucansucrase gene in *E. coli* DH5α or JM109 using a partial DNA library strategy with different vectors failed. Therefore, the C-terminal part was isolated by inverse PCR. The remaining fragment, located between the *Eco*RI/*Bgl*II and *Eco*RI/*Hind*III fragments, was isolated by PCR

techniques (figure 1D). The amplicons obtained were sequenced directly. To eliminate errors due to the PCR reaction, these fragments were sequenced for at least 4 times, using different clones per PCR reaction. Both DNA strands of the entire glucosyltransferase gene were sequenced twice. In this way the sequence of a 5.5 kb region of the *Lactobacillus reuteri* chromosomal DNA, containing the *gtfA* gene and its surroundings, were obtained.

The plasmids for expression of the glucosyltransferase gene in *E. coli* DH5α were constructed as described hereafter. A 4.8 kb fragment, containing the entire glucosyltransferase gene was generated by PCR, using the primers GTFpr3 (5'ACAACCACCA TGGAATTAGG TCGCACTGAT GTAAC3') (SEQ ID No. 8) and GTFpr4 (5'GCCAGCTGGA TCCGTCGACT AGTTTATTTT TGATCAAGCA TCTTACC3') (SEQ ID No. 9). Both primers contained suitable restriction enzyme recognition sites at their 5' ends (*Nco*I in GTFpr3 and *Bam*HI and *Sal*I in GTFpr4). Cloning of this PCR fragment in different vectors failed. Therefore, the strategy depicted in figure 2 was followed. Briefly, the PCR product was restricted with *Xba*I/*Pst*I and *Pst*I/*Bam*HI (figure 1; *Bam*HI site was introduced with GTFpr4). The resulting fragments (1503 bp and 2696 bp, respectively) were cloned separately in pBluescriptIISK$^+$ yielding pBXP1500 and pBPB2700. Ligation of the 2700 bp *Pst*I/*Sal*I fragment isolated from pBPB2700 in pBXP1500, digested with *Pst*I and *Sal*I, yielded pBGTF (7146 bp) in *E. coli* DH5α. Plasmid DNA of *E. coli* was isolated using the alkaline lysis method of Birnboim and Doly (1979) Nucleic. Acid Res. 7, 1513-1523 or with a Qiagen plamid kit following the instructions of the supplier. Cells of *E. coli* DH5α with pBGTF were harvested by centrifugation after 16h of growth. The pellet was washed with 50 mM sodium acetate buffer pH 5.5 containing 1 mM $CaCl_2$ and 1% (v/v) Tween-80 and the suspension was centrifuged again. Pelleted cells were resuspended in 50 mM sodium acetate buffer pH 5.5 containing 1 mM $CaCl_2$, 1% (v/v) Tween-80 and 7.2 mM β-mercaptoethanol. Cells were broken by sonication. Cell debris and intact cells were removed by centrifugation for 15 min at 4°C at 14,000 rpm in an Eppendorf centrifuge and the resulting cell free extract was used in the enzyme assays.

The glucosyltransferase activity was determined at 37°C by monitoring the release of fructose from sucrose or by measuring the amount of glucan produced using *E. coli* cell free extracts or *Lactobacillus reuteri* culture supernatant in reaction buffer (50 mM sodium acetate, 1 mM $CaCl_2$, 1% (v/v) Tween-80, 10 g/l sucrose, pH 8). Sucrose, glucose and fructose were determined using commercially available kits. For determination of the

molecular weight of the glucosyltransferase produced by *E. coli* or *L. reuteri*, SDS-PAGE was performed according to Laemmli (1970) Nature 227, 680-685. SDS-PAGE gels were stained using the PAS activity staining. Glucans were collected by precipitation with ethanol. ^{1}H-NMR spectroscopy (figure 6) and methylation analysis (table 1) were performed as described by van Geel-Schutten *et al.* (1999) Appl. Environ. Microbiol. 65, 3008-3014. The molecular weights of the glucans were determined by high performance size exclusion chromotography coupled on-line with a multi angle laser light scattering and a differential refractive index detector. After the first nucleotide sequencing of the obtained DNA two putative start codons leading to either a protein encoded by 3834 nucleotides (starting at nucleotide position 1670 of SEQ ID No. 1) or a protein encoded by 3753 nucleotides (starting at nucleotide position 1751 of SEQ ID No. 1) were identified. Both putative start codons were preceded by a putative ribosome binding site, GCAGG (located 4 base pairs upstream of nucleotide position 1670 of SEQ ID No. 1) or AGAAG (located 14 base pairs upstream of nucleotide position 1751 of SEQ ID No. 1), respectively. Depending on the potential start codon used, one of these glucosyltransferases comprised 1278 amino acids (starting at amino acid positions 504 of SEQ ID No. 2) (3834 nucleotides) and the other comprised 1251 amino acids (starting at amino acid position 531 of SEQ ID No. 2) (3753 nucleotides). The molecular weight (MW) deduced of the amino acid sequence of these glucosyltransferases was 143 and 140 kDa, respectively. The isoelectric point deduced of the amino acid sequence of these glucosyltranferases was 4.73 (for the higher MW protein) and 4.71 (for the lower MW protein), at pH 7, respectively. Surprisingly, the molecular weight of the purified protein from *Lactobacillus reuteri* indicated by SDS-PAGE was not approximately 140 kDa, but about 190 kDa. After repeating the nucleotide sequencing, it appeared that the above mentioned nucleotide forms did not represent the complete nucleotide sequence of the glucosyltransferase according to the invention, but were merely a part of the complete nucleotide sequence encoding the protein of the invention. The complete nucleotide sequence of the novel glucosyltransferase is represented in SEQ ID No. 1 and the amino acid sequence of said glucosyltransferase is shown in SEQ ID No. 2. All experiments were performed with both the complete nucleotide or amino acid sequence of the protein (SEQ ID No. 1 and 2, respectively) and the partial nucleotide or amino acid sequence mentioned above. The results of the experiments performed with the complete or partial amino acid sequence and the complete or partial nucleotide sequence mentioned above were identical indicating that the part of the glucosyltransferase represented by said partial nucleotide

and amino acid sequences is essential for the functionality of the glucosyltransferase according to the invention.

Table 1: Methylation analysis of the glucans produced by *Lactobacillus reuteri* strains and *E. coli* GTFA.

Type of glucosyl units	*Lactobacillus reuteri* strain 121	*Lactobacillus reuteri* strain 35-5	*E. coli* GTFA
Glc*p*-(1→	24%	25%	21%
→4)-Glc*p*-(1→	42%	43%	44%
→6)-Glc*p*-(1→	22%	21%	24%
→4,6)-Glc*p*-(1→	12%	11%	11%

Example 2: Adhesion experiments with *Lactobacillus* strains.

The adhesion of *Lactobacillus reuteri* strains to Caco-2 cell lines was determined as described below. Firstly, a bacterial suspension was prepared as follows. *L. reuteri* strains LB 121, 35-5 and *L. rhamnosus* LGG (a well known probiotic strain with good adhering properties) were cultured in MRS broth supplemented with 5 µl/ml of methyl-1,2-[^{3}H]-thymidine at 37°C for 18-20 h before the adhesion assays. The cultures were harvested by centrifugation, washed with phosphate buffered saline (PBS) and resuspended in PBS or PBS supplemented with 30 g/l sucrose (see Table 2) to a final density of about 2 x 10^9 cfu/ml. Prior to the adhesion assay, the cell suspensions in PBS with 30 g/l sucrose were incubated for 1 hour at 37°C, whereas the cell suspensions in PBS were kept on ice for 1 hour. After incubation at 37°C, the suspensions in PBS with sucrose were centrifuged and the cells were washed with and resuspended in PBS to a final density of about 2 x 10^9 cfu/ml.

Caco-2 cells were cultured as follows. Subcultures of Caco-2 cells (ATCC, code HTB 37, human colon adenocarcinoma), stored as frozen stock cultures in liquid nitrogen were used for the adhesion tests. The Caco-2 cells were grown in culture medium consisting of Dulbecco's modified Eagle medium (DMEM), supplemented with heat-inactivated foetal calf serum (10% v/v), non-essential amino acids (1% v/v), L-glutamine (2mM) and gentamicin (50 µg/ml). About 2,000,000 cells were seeded in 75cm^2 tissue culture flasks containing culture medium and cultured in a humidified incubator at 37°C in air containing 5% CO_2. Near confluent Caco-2 cell cultures were harvested by

trypsinisation and resuspended in culture medium. The number of cells was established using a Bürker-Türk counting chamber.

Table 2: Incubation of the different *Lactobacillus* strains prior to the adhesion assays.

Lactobacillus strain	Extra incubation	Polysaccharide produced	Group
reuteri 121	PBS sucrose, 37°C, 1 hr	glucan and fructan	As
reuteri 35-5	PBS sucrose, 37°C, 1 hr	glucan	Bs
reuteri K24	PBS sucrose, 37°C, 1 hr	none	Cs
reuteri 121	PBS on ice	none	D
reuteri DSM20016*	PBS on ice	none	E
rhamnosus GG	PBS on ice	none	F

* Type strain of *L. reuteri*

For the following experiments a Caco-2 monolayer transport system was used. Caco-2 cells cultured in a two-compartment transport system are commonly used to study the intestinal, epithelial permeability. In this system the Caco-2 cell differentiates into polarised columnar cells after reaching confluency. The Caco-2 system has been shown to simulate the passive and active transcellular tranport of electrolytes, sugars, amino acids and lipophilic compounds (K.M. Hillgren, A, Kato, R.T. Brochardt, Medical Research Reviews 15: 83-109, 1995; W.J. Dulfer, J.P. Groten, H.A.J. Govers, J. Lipid Res. 37: 950-961, 1996; E. Duizer, A.H. Penninks, W.H. Stenhuis and J.P. Groten, J. Contr. Rel. 49: 39- 49, 1997). Also, a clear correlation between the *in vivo* absorption and the permeability across the monolayers of Caco-2 cells has been reported (P. Artursson and J. Karlsson, Biochem. Biophys. Res. Commun. 75: 880-885, 1991). For the present transport studies, Caco-2 cells were seeded on semi-permeable filter inserts (12 wells Transwell plates, Costar) at ca. 100,000 cells per filter (growth area ± 1 cm^2 containing 2.5 ml culture medium). The cells on the insert were cultured for 17 to 24 days at 37°C in a humidified incubator containing 5% CO_2 in air. During this culture period the cells have been subjected to an enterocyte-like differentiation. Gentamycin was eliminated from the culture medium two days prior to the adhesion assays.

The adhesion assay was performed as follows. PBS was used as exposure medium. 25 µl of a bacterial suspension (2 x 10^9 cfu/ml) were added to 0.5 ml medium. The apical side of the Caco-2 monolayers was incubated with the bacterial suspensions for 1 hour at 37°C. After incubation, remaining fluid was removed and the cells were washed three

times with 1 ml PBS. Subsequently, the Caco-2 monolayers were digested overnight with 1 ml 0.1M NaOH, 1% SDS. The lysate was mixed with 10 ml Hionic Fluor scintillation liquid and the radioactivity was measured by liquid scintillation counting using a LKB/-Wallac scintillation counter. As a control, the radioactivity of the bacterial suspensions was measured. For each test group, the percentage of bacteria attached to the monolayers was calculated. All adhesion tests were performed in quadruple. In Table 3 the results of the bacterial adhesion test to Caco-2 cell lines are given. From the results can be concluded that the glucans and the fructans contribute to the adherence of *L. reuteri* to Caco-2 cell lines. This could indicate that *L. reuteri* strains producing EPS possess improved probiotic characteristics or that *L. reuteri* and its polysaccharides could function as an excellent symbiotic.

Table 3: The results of the bacterial adhesion test to Caco-2 cell lines.

Group (see Table 1)	% of bacteria bound to the monolayer
As	6.5
Bs	5.7
Cs	1.8
D	2.3
E	0.9
F	1.3

Example 3: Oxidation of glucans.

For TEMPO-mediated oxidation, a glucan produced as described above (dry weight 1 g, 6.15 mmol) was resuspended in 100 ml water. Next, 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO; 1% by weight compared to the polysaccharide (0.01 g, 0.065 mmol)) was added and resuspended in 20 min. Sodium bromide (0.75 g, 7.3 mmol) was added and the suspension was cooled to 0°C. The reaction can also be performed without bromide. A solution of hypochlorite (6 ml, 15% solution, 12.6 mmol) was adjusted to pH 10.0 with 3M HCl and cooled down to 0°C. This solution was added to the suspension of the polysaccharide and TEMPO. The course of the reaction was followed by monitoring the consumption of sodium hydroxide solution, which is equivalent to the formation of uronic acid. After 30 min, the amount of 0.1M NaOH consumed corresponded to the formation of 96% uronic acid, with respect to the available units having a primary

hydroxyl group. Thereafter, the solution was poured out in 96% ethanol (comprising 70% of the volume of the solution) causing the product to precipitate. The white precipitate was centrifuged, resuspended in ethanol/water (70/30 v/v) and centrifuged again. Next, the precipitate was resuspended in 96% ethanol and centrifuged. The obtained product was dried at reduced pressure. The uronic acid content was determined by means of the uronic acid assay according to Blumenkrantz and Abdoe-Hansen (Anal. Biochem., 54 (1973), 484). A calibration curve was generated using polygalacturonic acid (5, 10, 15 and 20 μg). With this calibration curve the uronic acid content in a sample of 20 μg of the product was determined. The obtained result was a content of 95% uronic acid with respect to available 6-hydroxymethyl groups, the with a yield of 95%.

For partial oxidation (about 6%), a glucan produced as described before (dry weight 7.5 g, 46.4 mmol) was resuspended in 50 ml water. Next, TEMPO (1% by weight compared to the polysaccharide (0.075 g, 0.5 mmol)) was added, resuspended in 20 min and cooled to 0°C. A solution of hypochlorite (2.8 ml, 15% solution, 5.9 mmol) was adjusted to pH 9.0 with 3M HCl and cooled down to 0°C. This solution was added to the suspension of the polysaccharide and TEMPO. Within 5 min the mixture became a solid gel.

Description of the figures

SEQ ID No. 2: The deduced amino acid sequence of glucosyltransferase A (GTFA) of *Lactobacillus reuteri*.

Figure 1: The strategy used for the isolation of the *gtfA* gene from *Lactobacillus reuteri* 121 chromosomal DNA.

Figure 2: The general principle of the construction of the recombinant plasmid with the *gtfA* gene. A PCR product containing the *gtfA* gene was digested with *Xba*I and *Pst*I and with *Pst*I and *Bam*HI. The *Xba*I/*Pst*I (depicted in white) was ligated into the multiple cloning site of pBluescriptIISK$^+$ in the same direction relatively to the *lacZ* promotor, resulting in pBXP1500. The *Pst*I/*Bam*HI part (depicted in grey) was ligated into the multiple cloning site of pBluescriptIISK$^+$ in the opposite direction relatively to the *lacZ* promotor, resulting in pBPB2700. pBXP1500 was used as a vector for subcloning the C-terminal part of the *gtfA*. pBPB2700 was digested with *Pst*I and *Sal*I and ligated into pBXP1500, also digested with *Pst*I and *Sal*I. The resulting plasmid, pBGTF, contained the entire *gtfA* in the same direction relative to the *lacZ* promotor. The ✂ sign indicates the restriction.

Figure 3: The nucleotide and deduced amino acid sequence of *gtfA* of *Lactobacillus reuteri*. The start codon is shown in bold. The putative ribosomal binding site (RBS) is also shown in bold. The nucleotides in italic and underlined upstream of the start codon indicate the -10 region (Pribnow box) and the -35 region. The inverted repeats (transcription termination) after the stop codon are underlined. The * sign indicates the stop codon.

Figure 4: Alignment of catalytic cores of alternansucrase (ASR) of *Leuconostoc mesenteroides* strain NRRL B-1355 dextransucrase (DSRS) of *Leuconostoc mesenteroides* strain NRRL B-512F, glucosyltransferase-D (GTFD) of *Streptococcus mutans* GS5, glucosyltransferase-A of *Lactobacillus reuteri* and amylosucrase (AS) of *Neisseria polysaccharea*. * indicates identical or conserved residues in all sequences); ---, gap in the sequence; **AA** amino acids which are conserved in all other glucosyltranferases but not in GTFA; ⇓, putative catalytic residues; •, putative calcium binding sites; ♦, putative residues stabilising the transition state; ∇, residues possibly playing a role in binding of acceptor molecules and in the transfer of the glucosyl residue; ◊, putative chloride binding sites; **-Ex-**, localisation of β-strands; **-Hx-**, localisation of α-helices according to Mac Gregor *et al.* (1996) FEBS Lett. 378, 262-266. The numbering of the amino acids of the glucosyltransferase-A of *Lactobacillus reuteri* corresponds to the positions of these amino acids in the amino acid sequence 531-1781 of amino acid sequence SEQ ID No. 2, when the amino acid sequence 531-1781 is renumbered 1-1251.

Figure 5: Dendrogram of glucansucrases of lactic acid bacteria. The horizontal distances are a measure for the difference at the amino acid sequence level. 10% difference is indicated by the upper bar. Bootstrap values (in percentages) are given at the root of each branch.

Figure 6: 500-MHz ^{1}H-NMR spectra of the glucan produced by *Lactobacillus reuteri* GTFA (A) and by *E. coli* GTFA (B), recorded in D_2O at 80°C.

Claims

1. A protein having glucosyltransferase activity comprising an amino acid sequence, exhibiting at least 50% amino acid identity, as determined by the BLAST algorithm, with the amino acid sequence 531-1781 of SEQ ID No. 2 or a part thereof having at least 15 contiguous amino acids which are identical to the corresponding part of the amino acid sequence 531-1781 of SEQ ID No. 2.

2. A protein according to claim 1 comprising an amino acid sequence, exhibiting at least 60%, preferably at least 70%, amino acid identity with the amino acid sequence 531-1781 of SEQ ID No. 2.

3. A protein according to claim 1 or 2, exhibiting at least 50% amino acid identity, as determined by the BLAST algorithm, with the amino acid sequence of SEQ ID No. 2 or a part thereof having at least 15 contiguous amino acids which are identical to the corresponding part of the amino acid sequence of SEQ ID No.2.

4. A protein according to claim 3, exhibiting at least 60%, preferably at least 70%, amino acid identity with the amino acid sequence of SEQ ID No. 2.

5. A protein according to any one of claims 1-4, comprising an amino acid sequence of at least 200 amino acids exhibiting at least 55%, preferably at least 65%, amino acid identity with the corresponding part of the amino acid sequence 972-1514 of SEQ ID No. 2.

6. A protein according to any one of claims 1-5, comprising an amino acid sequence of at least 100 amino acids exhibiting at least 50%, preferably at least 60%, amino acid identity with the corresponding part of the amino acid sequence 1515-1781 of SEQ ID No. 2.

7. A protein according to any one of claims 1-6, comprising at least one of the amino acids Pro-1026, Ile-1029, Met-1034, Asn-1035, Ser-1136, Ala-1143, Ile-1170, Leu-1223, Ala-1413, Val-1418, Ala-1428, Leu-1442 of the amino acid sequence of SEQ ID No. 2.

8. A protein according to any one of the preceding claims which, in the presence of sucrose, produces a glucan having 38-48% 4-linked anhydroglucose units, 17-28% 6-linked anhydroglucose units, and 7-20% 4,6-linked anhydroglucose units.

9. A protein according to any one of the preceding claims which is a recombinant protein.

10. A nucleotide sequence encoding a protein according to any one of the preceding claims.

11. A nucleic acid construct comprising the nucleic acid sequence of claim 10, operationally linked to an expression-regulating nucleic acid sequence.

12. A recombinant host cell containing one or more copies of the nucleic acid construct according to claim 11.

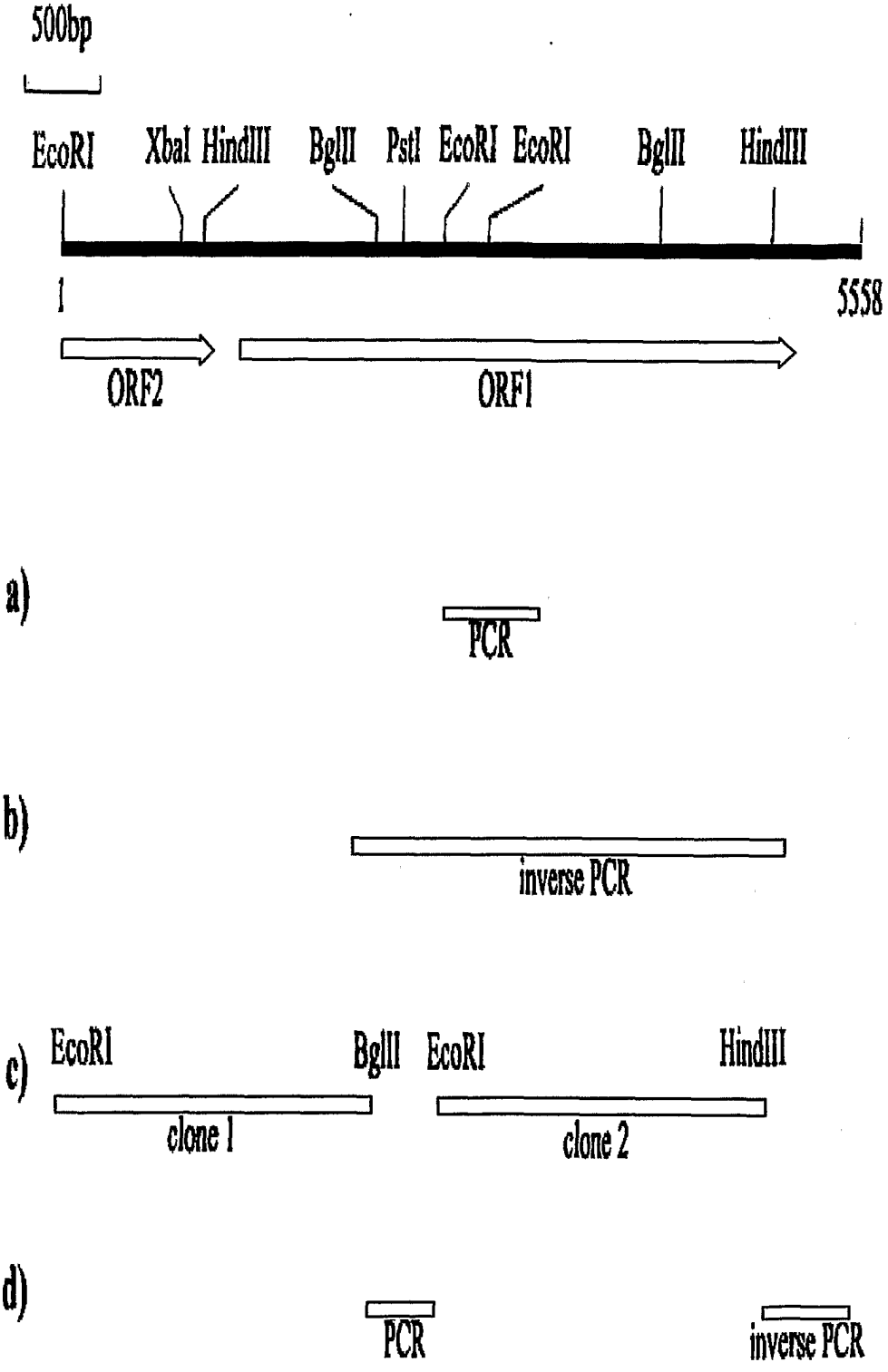
13. A process of producing a protein of interest, such as a glucosyltransferase, comprising culturing a host cell according to claim 12 in a culture medium, and recovering the protein from the culture medium or the cell free extract.

14. A process of producing an oligosaccharide or polysaccharide of interest, using a protein according to any one of claims 1-9, or a host cell according to claim 12.

15 A chemically modified glucan, which is obtained by 2,3-oxidation, 6-oxidation, phosphorylation, acylation, hydroxyalkylation, carboxymethylation, aminoalkylation of one or more anhydroglucose units of a glucan comprising at least 20, up to about 100,000 α-anhydroglucose units, 38-48% of which are 4-linked anhydroglucose units, 17-28% are 6-linked anhydroglucose units, and 7-20% are 4,6-linked anhydroglucose units.

16 Use of a *Lactobacillus* strain capable of producing a glucan comprising at least 20, up to about 100,000 α anhydroglucose units, 38-48% of which are 4-linked anhydroglucose units, 17-28% are 6-linked anhydroglucose units, and 7-20% are 4,6-linked anhydroglucose units with sucrose as a substrate and optionally a fructan as a probiotic or symbiotic.

Fig 1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XbaI
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PstI
EcoRI
EcoRI
BglII
HindIII
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ORF2
ORF1
a)
PCR
b)
inverse PCR
c)
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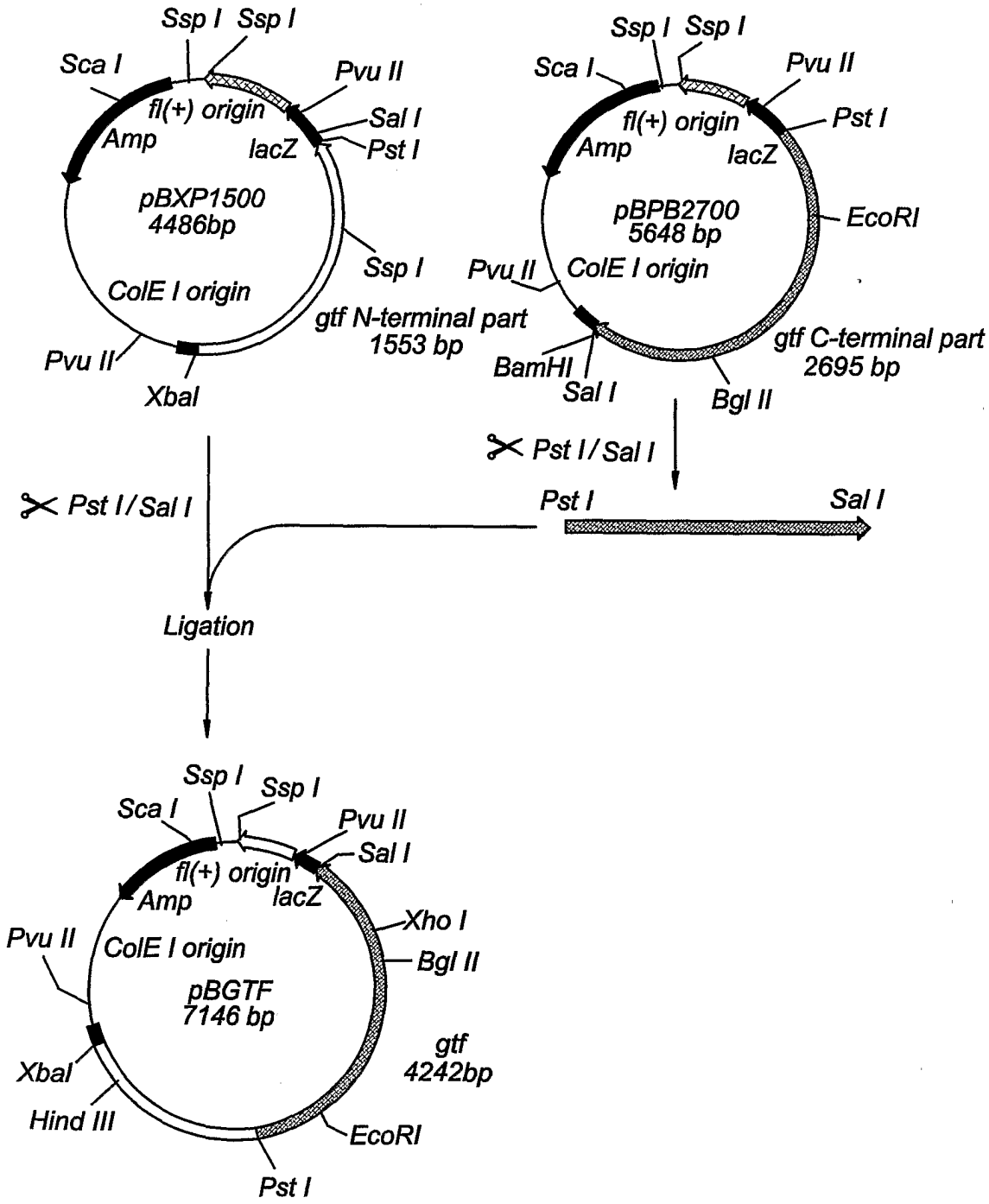
EcoRI
HindIII
clone 1
clone 2
d)
PCR
inverse PCR

Fig 2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Bgl II
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Pst I/Sal I
Pst I
Sal I
Ligation
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Pvu II
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gtf
4242bp
XbaI
Hind III
EcoRI
Pst I

Fig.3.1

```
1     ATACATATTTTGGGCTTCTTTTTTTGTTTAAAACTGTAAATTTGAATTTTATTTGAAAAA
1       Y  I  F  W  A  S  F  F  V  *  N  C  K  F  E  F  Y  L  K  K
                        -35                                  -10
61    ATTTTGGCTAGAATTTGAAAATTCCCTTTGAAAAAATAAAACATCATAGTATTATAATAC
21      F  W  L  E  F  E  N  S  L  *  K  N  K  T  S  *  Y  Y  N  T
                                  RBS        start
121   CGATAATCAAATTGTTTATTTTGATATGAAGGAGATTAAAATGGAAATAAAGAAACATTT
41      D  N  Q  I  V  Y  F  D  M  K  E  I  K  M  E  I  K  K  H  F

181   TAAGTTGTACAAAAGCGGCAAACAATGGGTAACAGCAGCAGTGGCTACCGTTGCCGTTTC
61      K  L  Y  K  S  G  K  Q  W  V  T  A  A  V  A  T  V  A  V  S

241   AACTGCGCTTCTTTACGGAGGAGTTGCGCATGCTGACCAACAAGTTCAGCAAGCTTCCAC
81      T  A  L  L  Y  G  G  V  A  H  A  D  Q  Q  V  Q  Q  A  S  T

301   GACTCAAGACCAAACTTCTACCGTAAATAATGATACTGATAAAACAGTAGCTTTAGATAC
101     T  Q  D  Q  T  S  T  V  N  N  D  T  D  K  T  V  A  L  D  T

361   TAATACTGACCAGTCAGCTCAAACAACTGATAAAAAACAAGTAGTATCAAATACTAACCA
121     N  T  D  Q  S  A  Q  T  T  D  K  K  Q  V  V  S  N  T  N  Q

421   AAGCAAAACTGATGACACTTCAACAGCTGATAAGAATTCTACTTCTACACCTGTTTCTGT
141     S  K  T  D  D  T  S  T  A  D  K  N  S  T  S  T  P  V  S  V

481   TTTGCCATCTAATAATACTGAAAAACAAGCTAAAAATTATAATGAGCAAGACAAAGGAAA
161     L  P  S  N  N  T  E  K  Q  A  K  N  Y  N  E  Q  D  K  G  N

541   CTATGGGAATATTGATACTGCTTACTTTAGCAATAATCAATTGCATGTTTCAGGATGGAA
181     Y  G  N  I  D  T  A  Y  F  S  N  N  Q  L  H  V  S  G  W  N

601   TGCAACGAACGCATCTCAAGGAACAAACAGTCGACAAATCATTGTGCGTGATATCACAAC
201     A  T  N  A  S  Q  G  T  N  S  R  Q  I  I  V  R  D  I  T  T

661   CAATAATGAATTAGGTCGCACTGATGTAACAAACAATGTTGCACGCCCAGACGTTAAGAA
221     N  N  E  L  G  R  T  D  V  T  N  N  V  A  R  P  D  V  K  N
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Fig.3.2

```
721   TGTTCATAATGTTTATAACGCTGATAATTCTGGATTTGATGTTAATGTCAATATTGACTT
241     V  H  N  V  Y  N  A  D  N  S  G  F  D  V  N  V  N  I  D  F

781   TAGCAAGATGAAAGATTATCGGGATTCAATTGAAATTGTTAGTCGATACAGTGGAAACGG
261     S  K  M  K  D  Y  R  D  S  I  E  I  V  S  R  Y  S  G  N  G

841   TAAATCTGTTGACTGGTGGTCCCAACCGATCACTTTTGACAAAAACAACTATGCTTATCT
281     K  S  V  D  W  W  S  Q  P  I  T  F  D  K  N  N  Y  A  Y  L

901   TGATACATTTGAAGTGAAAAATGGCGAATTACATGCAACCGGATGGAATGCTACTAATAG
301     D  T  F  E  V  K  N  G  E  L  H  A  T  G  W  N  A  T  N  S

961   TGCGATTAACTATAATCACCATTTTGTGATTTTGTTTGATCAAACGAATGGTAAAGAAGT
321     A  I  N  Y  N  H  H  F  V  I  L  F  D  Q  T  N  G  K  E  V

1021  AGCACGACAAGAAGTTCGTGAAGGTCAATCACGCCCAGATGTTGCTAAGGTATATCCACA
341     A  R  Q  E  V  R  E  G  Q  S  R  P  D  V  A  K  V  Y  P  Q

1081  AGTAGTTGGTGCTGCCAACTCAGGCTTTAATGTGACATTTAATATCAGTGATTTAGATTA
361     V  V  G  A  A  N  S  G  F  N  V  T  F  N  I  S  D  L  D  Y

1141  TACTCACCAGTACCAAGTTCTTAGTCGTTACAGCAATTCTGATAATGGCGAAGGTGATAA
381     T  H  Q  Y  Q  V  L  S  R  Y  S  N  S  D  N  G  E  G  D  N

1201  CGTTACCTACTGGTTTAATCCACAATCCATTGCTCCTGCTAATCAAAGTAACCAGGGTTA
401     V  T  Y  W  F  N  P  Q  S  I  A  P  A  N  Q  S  N  Q  G  Y

1261  TCTAGACTCATTTGATATTAGTAAAAATGGTGAAGTAACAGTAACTGGATGGAACGCTAC
421     L  D  S  F  D  I  S  K  N  G  E  V  T  V  T  G  W  N  A  T

1321  TGACTTGTCAGAATTACAAAACAACCATTATGTGATTCTATTTGATCAGACAGCAGGCAA
441     D  L  S  E  L  Q  N  N  H  Y  V  I  L  F  D  Q  T  A  G  K
```

Fig.3.3

```
1381  ACAAGTTGCATCTGCTAAAGCTGATTTAATTTCACGTCCAGATGTTGCTAAAGCTTATCC
461     Q  V  A  S  A  K  A  D  L  I  S  R  P  D  V  A  K  A  Y  P

1441  AACAGTAAAAACAGCTACAAATTCTGGCTTCAAGGTAACATTTAAGGTTAATAACTTACA
481     T  V  K  T  A  T  N  S  G  F  K  V  T  F  K  V  N  N  L  Q

1501  ACCGGGTCACCAATACAGCGTTGTAAGTCGTTTCTCTGCCGATGAAAATGGTAATGGTAA
501     P  G  H  Q  Y  S  V  V  S  R  F  S  A  D  E  N  G  N  G  N

1561  TGATAAGCGCCATACAGATTACTGGTTTAGTCCAGTAATATTAAACCAGACTGCTTCAAA
521     D  K  R  H  T  D  Y  W  F  S  P  V  I  L  N  Q  T  A  S  N

1621  CATTGATACTATTACAATGACATCTAATGGTTTACATATTGCAGGTTGGATGGCAAGTGA
541     I  D  T  I  T  M  T  S  N  G  L  H  I  A  G  W  M  A  S  D

1681  TAACTCAATTAATGAAACAACTCCATACGCTATTATCCTCAATAATGGAAAAGAAGTTAC
561     N  S  I  N  E  T  T  P  Y  A  I  I  L  N  N  G  K  E  V  T

1741  TCGTCAAAAGATGAGCTTAACCGCCCGTCCAGATGTAGCAGCAGTATATCCTTCACTTTA
581     R  Q  K  M  S  L  T  A  R  P  D  V  A  A  V  Y  P  S  L  Y

1801  TAATAGTGCTGTTAGTGGTTTTGACACTACTATTAAATTGACTAATGATCAATATCAAGC
601     N  S  A  V  S  G  F  D  T  T  I  K  L  T  N  D  Q  Y  Q  A

1861  GCTTAATGGCCAATTACAAGTATTGTTACGTTTTTCTAAAGCTGCTGATGGTAATCCAAG
621     L  N  G  Q  L  Q  V  L  L  R  F  S  K  A  A  D  G  N  P  S

1921  TGGTGATAATACTGTAACTGATCAATTTAGTAAAAATTATGCAACTACTGGTGGAAACTT
641     G  D  N  T  V  T  D  Q  F  S  K  N  Y  A  T  T  G  G  N  F

1981  TGACTATGTAAAAGTAAATGGTAATCAAGTTGAATTTAGTGGTTGGCACGCAACTAACCA
661     D  Y  V  K  V  N  G  N  Q  V  E  F  S  G  W  H  A  T  N  Q
```

Fig.3.4

```
2041  ATCAAATGATAAAGATTCACAATGGATTATTGTTTTAGTTAATGGTAAGGAAGTAAAGCG
681     S  N  D  K  D  S  Q  W  I  I  V  L  V  N  G  K  E  V  K  R

2101  TCAATTAGTTAATGATACTAAAGAGGGAGCTGCTGGCTTCAACCGAAACGATGTCTACAA
701     Q  L  V  N  D  T  K  E  G  A  A  G  F  N  R  N  D  V  Y  K

2161  AGTAAATCCAGCTATTGAAAACAGTTCTATGTCTGGATTCCAAGGCATTATTACTTTACC
721     V  N  P  A  I  E  N  S  S  M  S  G  F  Q  G  I  I  T  L  P

2221  TGTGACAGTTAAAAACGAAAATGTCCAACTTGTTCATCGGTTTAGTAACGATGTGAAGAC
741     V  T  V  K  N  E  N  V  Q  L  V  H  R  F  S  N  D  V  K  T

2281  TGGTGAAGGTAACTATGTTGATTTCTGGTCAGAGCTAATGCCTGTTAAGGATAGCTTCCA
761     G  E  G  N  Y  V  D  F  W  S  E  L  M  P  V  K  D  S  F  Q

2341  AAAGGGGAATGGCCCACTTAAGCAATTTGGCTTACAAACTATTAACGGTCAACAATATTA
781     K  G  N  G  P  L  K  Q  F  G  L  Q  T  I  N  G  Q  Q  Y  Y

2401  TATTGACCCAACAACTGGTCAACCACGTAAGAATTTCTTATTACAAAGTGGAAATAATTG
801     I  D  P  T  T  G  Q  P  R  K  N  F  L  L  Q  S  G  N  N  W

2461  GATTTACTTTGATAGTGATACTGGTGTGGGTACTAATGCACTTGAATTACAATTTGCAAA
821     I  Y  F  D  S  D  T  G  V  G  T  N  A  L  E  L  Q  F  A  K

2521  GGGAACTGTTTCATCTAATGAACAATACCGTAACGGTAATGCAGCTTACAGTTATGATGA
841     G  T  V  S  S  N  E  Q  Y  R  N  G  N  A  A  Y  S  Y  D  D

2581  CAAGAGTATCGAAAATGTAAATGGTTACTTAACAGCAGATACATGGTACCGTCCAAAACA
861     K  S  I  E  N  V  N  G  Y  L  T  A  D  T  W  Y  R  P  K  Q

2641  GATCTTAAAGGATGGAACTACCTGGACTGACTCAAAAGAAACAGATATGCGACCAATCTT
881     I  L  K  D  G  T  T  W  T  D  S  K  E  T  D  M  R  P  I  L

2701  GATGGTATGGTGGCCTAATACTCTTACCCAAGCATACTACCTTAATTACATGAAACAACA
```

Fig.3.5

```
901     M  V  W  W  P  N  T  L  T  Q  A  Y  Y  L  N  Y  M  K  Q  H

2761  TGGTAATTTATTACCATCTGCTTTACCATTCTTTAATGCGGATGCTGATCCTGCAGAATT
921     G  N  L  L  P  S  A  L  P  F  F  N  A  D  A  D  P  A  E  L

2821  AAATCATTATTCCGAAATTGTGCAACAAAATATTGAAAAACGAATTAGTGAAACCGGAAA
941     N  H  Y  S  E  I  V  Q  Q  N  I  E  K  R  I  S  E  T  G  N

2881  TACTGATTGGTTACGTACTTTAATGCACGATTTTGTTACTAACAATCCGATGTGGAATAA
961     T  D  W  L  R  T  L  M  H  D  F  V  T  N  N  P  M  W  N  K

2941  GGATAGTGAAAATGTTAACTTTAGTGGTATTCAATTCCAAGGCGGATTCTTAAAGTATGA
981     D  S  E  N  V  N  F  S  G  I  Q  F  Q  G  G  F  L  K  Y  E

3001  AAACTCAGATTTAACGCCTTATGCTAACTCTGATTATCGCTTACTTGGTCGGATGCCAAT
1001    N  S  D  L  T  P  Y  A  N  S  D  Y  R  L  L  G  R  M  P  I

3061  CAATATTAAGGATCAAACATATCGGGGACAAGAATTCCTACTTGCTAACGATATTGATAA
1021    N  I  K  D  Q  T  Y  R  G  Q  E  F  L  L  A  N  D  I  D  N

3121  CTCTAATCCTGTTGTTCAAGCAGAACAATTAAACTGGTTATACTATCTCTTGAACTTTGG
1041    S  N  P  V  V  Q  A  E  Q  L  N  W  L  Y  Y  L  L  N  F  G

3181  AACGATCACAGCTAATAATGATCAAGCTAATTTTGATTCTGTACGGGTAGATGCACCGGA
1061    T  I  T  A  N  N  D  Q  A  N  F  D  S  V  R  V  D  A  P  D

3241  TAATATTGATGCCGATCTTATGAATATCGCTCAGGACTACTTTAATGCTGCATATGGTAT
1081    N  I  D  A  D  L  M  N  I  A  Q  D  Y  F  N  A  A  Y  G  M

3301  GGACTCAGATGCTGTCTCAAATAAGCATATTAATATTCTTGAAGACTGGAATCATGCTGA
1101    D  S  D  A  V  S  N  K  H  I  N  I  L  E  D  W  N  H  A  D

3361  TCCGGAATACTTTAATAAGATCGGAAATCCACAATTGACAATGGATGATACTATTAAGAA
1121    P  E  Y  F  N  K  I  G  N  P  Q  L  T  M  D  D  T  I  K  N
```

Fig.3.6

```
3421  TTCCCTGAATCATGGGCTTTCAGATGCAACTAATCGTTGGGGATTAGATGCAATTGTTCA
1141    S  L  N  H  G  L  S  D  A  T  N  R  W  G  L  D  A  I  V  H

3481  TCAGTCATTAGCTGATCGTGAAAATAATTCCACGGAAAATGTTGTAATTCCTAATTACAG
1161    Q  S  L  A  D  R  E  N  N  S  T  E  N  V  V  I  P  N  Y  S

3541  TTTCGTTCGGGCTCACGATAATAATTCTCAAGATCAAATTCAAAATGCTATTCGTGATGT
1181    F  V  R  A  H  D  N  N  S  Q  D  Q  I  Q  N  A  I  R  D  V

3601  AACAGGCAAAGATTACCATACTTTCACTTTTGAAGATGAGCAAAAGGGTATTGATGCGTA
1201    T  G  K  D  Y  H  T  F  T  F  E  D  E  Q  K  G  I  D  A  Y

3661  CATTCAAGATCAAAATTCAACAGTGAAGAAATATAACCTTTATAATATTCCGGCTTCATA
1221    I  Q  D  Q  N  S  T  V  K  K  Y  N  L  Y  N  I  P  A  S  Y

3721  CGCAATTCTTTTAACTAACAAGGATACAATTCCACGTGTATACTATGGTGACTTGTATAC
1241    A  I  L  L  T  N  K  D  T  I  P  R  V  Y  Y  G  D  L  Y  T

3781  TGATGGTGGCCAATACATGGAACATCAAACACGTTACTATGATACTTTAACGAACCTGCT
1261    D  G  G  Q  Y  M  E  H  Q  T  R  Y  Y  D  T  L  T  N  L  L

3841  TAAATCACGAGTTAAGTATGTTGCCGGTGGCCAATCAATGCAAACAATGAGCGTTGGCGG
1281    K  S  R  V  K  Y  V  A  G  G  Q  S  M  Q  T  M  S  V  G  G

3901  CAATAATAACATTTTAACTAGTGTTCGTTATGGTAAAGGTGCGATGACAGCTACTGATAC
1301    N  N  N  I  L  T  S  V  R  Y  G  K  G  A  M  T  A  T  D  T

3961  TGGTACTGATGAAACCAGAACACAAGGTATTGGGGTTGTTGTAAGTAATACGCCAAATCT
1321    G  T  D  E  T  R  T  Q  G  I  G  V  V  V  S  N  T  P  N  L

4021  AAAGCTAGGTGTCAACGATAAAGTAGTTCTTCATATGGGAGCTGCGCACAAGAACCAACA
1341    K  L  G  V  N  D  K  V  V  L  H  M  G  A  A  H  K  N  Q  Q
```

Fig.3.7

```
4081  ATATCGGGCAGCCGTGTTGACGACAACTGATGGAGTCATTAATTATACTTCTGATCAAGG
1361    Y  R  A  A  V  L  T  T  T  D  G  V  I  N  Y  T  S  D  Q  G

4141  GGCACCGGTTGCAATGACTGACGAGAACGGTGATCTATACTTATCTAGTCATAACCTAGT
1381    A  P  V  A  M  T  D  E  N  G  D  L  Y  L  S  S  H  N  L  V

4201  TGTTAATGGTAAAGAAGAAGCAGATACAGCTGTTCAAGGTTATGCTAACCCTGATGTTTC
1401    V  N  G  K  E  E  A  D  T  A  V  Q  G  Y  A  N  P  D  V  S

4261  AGGATATCTTGCTGTATGGGTACCAGTTGGAGCAAGTGATAACCAAGATGCTCGAACTGC
1421    G  Y  L  A  V  W  V  P  V  G  A  S  D  N  Q  D  A  R  T  A

4321  TCCATCTACTGAAAAGAATAGTGGTAACTCTGCATACAGAACAAATGCTGCTTTTGATTC
1441    P  S  T  E  K  N  S  G  N  S  A  Y  R  T  N  A  A  F  D  S

4381  AAATGTTATTTTTGAAGCCTTTTCTAACTTTGTCTATACACCAACAAAGGAAAGTGAACG
1461    N  V  I  F  E  A  F  S  N  F  V  Y  T  P  T  K  E  S  E  R

4441  TGCTAATGTTCGAATTGCCCAAAATGCTGATTTCTTTGCTTCATTAGGTTTTACTTCTTT
1481    A  N  V  R  I  A  Q  N  A  D  F  F  A  S  L  G  F  T  S  F

4501  CGAGATGGCGCCACAATATAATTCAAGTAAAGATCGCACATTCCTAGATTCAACAATTGA
1501    E  M  A  P  Q  Y  N  S  S  K  D  R  T  F  L  D  S  T  I  D

4561  TAACGGATATGCGTTTACTGATCGTTATGATCTTGGAATGAGTGAGCCTAATAAGTACGG
1521    N  G  Y  A  F  T  D  R  Y  D  L  G  M  S  E  P  N  K  Y  G

4621  AACAGATGAAGATCTACGTAATGCCATTCAAGCGCTCCATAAAGCTGGCTTACAAGTAAT
1541    T  D  E  D  L  R  N  A  I  Q  A  L  H  K  A  G  L  Q  V  M

4681  GGCGGATTGGGTTCCTGACCAAATCTATAACCTTCCTGGAAAAGAAGTTGCTACAGTCAC
1561    A  D  W  V  P  D  Q  I  Y  N  L  P  G  K  E  V  A  T  V  T

4741  TCGAGTAGATGATCGTGGTAATGTATGGAAAGATGCTATCATTAATAATAATCTGTATGT
1581    R  V  D  D  R  G  N  V  W  K  D  A  I  I  N  N  N  L  Y  V
```

Fig.3.8

```
4801  TGTTAATACTATTGGTGGTGGCGAATACCAGAAGAAGTATGGTGGAGCATTCCTCGATAA
1601    V  N  T  I  G  G  G  E  Y  Q  K  K  Y  G  G  A  F  L  D  K

4861  GTTACAAAAACTTTATCCTGAAATCTTCACAAAGAAGCAAGTTTCAACTGGTGTTGCTAT
1621    L  Q  K  L  Y  P  E  I  F  T  K  K  Q  V  S  T  G  V  A  I

4921  TGATCCTTCACAAAAGATAACTGAATGGTCAGCAAAATACTTTAATGGAACAAACATTCT
1641    D  P  S  Q  K  I  T  E  W  S  A  K  Y  F  N  G  T  N  I  L

4981  CCATCGTGGTTCTGGTTATGTACTAAAAGCTGATGGTGGTCAATACTACAACTTAGGTAC
1661    H  R  G  S  G  Y  V  L  K  A  D  G  G  Q  Y  Y  N  L  G  T

5041  TACTACAAAGCAATTCTTGCCAATTCAATTAACTGGTGAAAAGAAACAAGGAAATGAAGG
1681    T  T  K  Q  F  L  P  I  Q  L  T  G  E  K  K  Q  G  N  E  G

5101  CTTTGTTAAGGGTAATGATGGAAATTACTACTTCTATGACTTAGCAGGTAATATGGTTAA
1701    F  V  K  G  N  D  G  N  Y  Y  F  Y  D  L  A  G  N  M  V  K

5161  GAATACCTTTATTGAAGATAGTGTTGGCAACTGGTACTTCTTTGACCAAGATGGTAAGAT
1721    N  T  F  I  E  D  S  V  G  N  W  Y  F  F  D  Q  D  G  K  M

5221  GGTTGAAAATAAACATTTCGTTGATGTTGATTCTTATGGTGAAAAAGGTACTTACTTCTT
1741    V  E  N  K  H  F  V  D  V  D  S  Y  G  E  K  G  T  Y  F  F

5281  CTTGAAGAATGGTGTATCATTCCGTGGGGGATTAGTGCAAACTGACAATGGTACTTATTA
1761    L  K  N  G  V  S  F  R  G  G  L  V  Q  T  D  N  G  T  Y  Y

5341  CTTTGATAATTATGGAAAGATGGTACGTAATCAAACTATTAATGCAGGTGCCATGATTTA
1781    F  D  N  Y  G  K  M  V  R  N  Q  T  I  N  A  G  A  M  I  Y

5401  TACCTTAGATGAAAACGGTAAGCTTATAAAGGCTAGTTATAATTCAGATGCCGAATATCC
1801    T  L  D  E  N  G  K  L  I  K  A  S  Y  N  S  D  A  E  Y  P
```

Fig.3.9

```
5461  AACTTCAACTGATGTTGGTAAGATGCTTGATCAAAATAAACTATAAATGGAAATAATTAG
1821    T  S  T  D  V  G  K  M  L  D  Q  N  K  L  *  M  E  I  I  S

5521  CTGATTTCCGTTTCTTAGAATCGAAAGATTTAATAACTGGGGTTAAAACGGCCCTACAAA
1841    *  F  P  F  L  R  I  E  R  F  N  N  W  G  *  N  G  P  T  K

5581  ATCTGATATTGATATAGAGATATTATTTCCTATATCAATATCAGATTTTTGCTTTTTATA
1861    S  D  I  D  I  E  I  L  F  P  I  S  I  S  D  F  C  F  L  *

5641  AAATTGATTGTGACTAATAAGAATCCGGAAGATAACGTTGTTGTTATATCAGTGGATTTA
1881    N  *  L  *  L  I  R  I  R  K  I  T  L  L  L  Y  Q  W  I  *

5701  AGCAACATGAATTAATTGAAGATGACGGCAATGATTAAAAGTCGGTCTGATGATTATTGA
1901    A  T  *  I  N  *  R  *  R  Q  *  L  K  V  G  L  M  I  I  D

5761  TGTATTACTAGTATTTGGTTTTTATCATTTATATTTTTACTGTTATTGGTGTCATATATT
1921    V  L  L  V  F  G  F  Y  H  L  Y  F  Y  C  Y  W  C  H  I  F

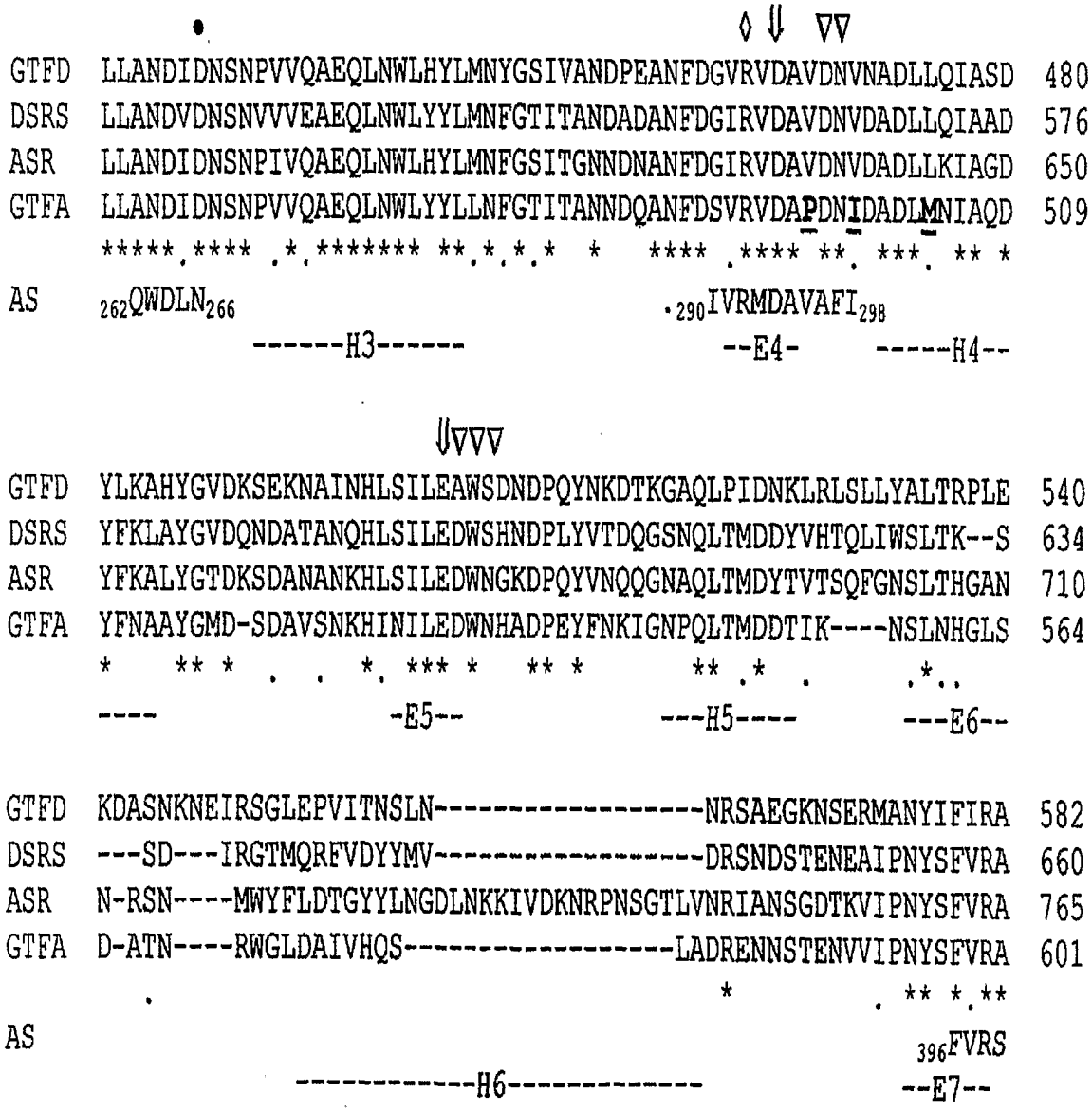
5821  CCACAATAACAGTAAAGGTATATATGCTAGTTTATTTTTTAAGTAATTATAATATTCTGA
1941    H  N  N  S  K  G  I  Y  A  S  L  F  F  K  *  L  *  Y  S  D

5881  TTATAATTTGGAAATATTCGCTTTTAGCAAAAAGGTAGTAAACAGATCAGAATCGTCATT
1961    Y  N  L  E  I  F  A  F  S  K  K  V  V  N  R  S  E  S  S  F

5941  CTGCTTTTCTACTACTAAAAGTCTGTTTTAAATTCTAAACTAAAATAGGCTAAACACTGA
1981    C  F  S  T  T  K  S  L  F  *  I  L  N  *  N  R  L  N  T  D

6001  TGTTTATCATTTATATTTTTACTGTT
2001    V  Y  H  L  Y  F  Y  C
```

Fig.4.1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D-ATN----RWGLDAIVHQS----------LADRENNSTENVVIPNYSFVRA 601
AS
396FVRS
----------H6----------
--E7--

Fig.4.2

```
GTFD  HDSEVQTVIAKIIKAQINPKTDGLTFTLDELKQAFKIYNEDM--RQAKKKYTQSNIPTAY  640
DSRS  HDSEVQTVIAQIVSDLYPDVENSLAPTTEQLAAAFKVYNEDE--KLADKKYTQYNMASAY  718
ASR   HDYDAQDPIRKAMIDHGIIKNMQDTFTFDQLAQGMEFYYKDQENPSGFKKYNDYNLPSAY  825
GTFA  HDNNSQDQIQNAIRDVTGKD--YHTFTFEDEQKGIDAYIQDQ-N-STVKKYNLYNIPASY  657
      **   * *   .          . * .       * *      ***.  *. ..*
AS    HD401
                                                        ----H7-

GTFD  ALMLSNKDSITRLYYGDMYSDDGQYMATKSPYYDAIDTLLKARIKYAAGGQDMKITYVEG  700
DSRS  AMLLTNKDTVPRVYYGDLYTDDGQYMATKSPYYDAINTLLKARVQYVAGGQ---------  769
ASR   AMLLTNKDTVPRVYYGDMYLEGGQYMEKGTIYNPVISALLKARIKYVSGGQTMATDSSGK  885
GTFA  AILLTNKDTIPRVYYGDLYTDGGQYMEHQTRYYDTLTNLLKSRVKYVAGGQSMQTMSVG-  716
      *..*.***.. *.****.* . ****   . *   .  ***.*..* .***
AS          488GLPRIYLGD496
      H7-       --E8-                  -------H8------

GTFD  DKSHMDWDYTGVLTSVRYGTGANEATDQGSEATK----TQGMAVITSNNPSLKLNQNDKV  756
DSRS  ---SMSVDSNDVLTSVRYGKDAMTASDTGTSETR----TEGIGVIVSNNAELQLEDGHTV  822
ASR   DL---KDGETDLLTSVRFGKGIMTSDQTTTQDNSQDYKNQGIGVIVGNNPDLKLNNDKTI  942
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DSRS  TLHMGAAHKNQAYRALLSTTADGLAYYDTDENAPVAYTDAN----------GDLIFT  869
ASR   TLHMGKAHKNQLYRALVLSNDSGIDVYDSDDKAPTLRTNDNGDLIFHKTNTFVKQDGTII 1002
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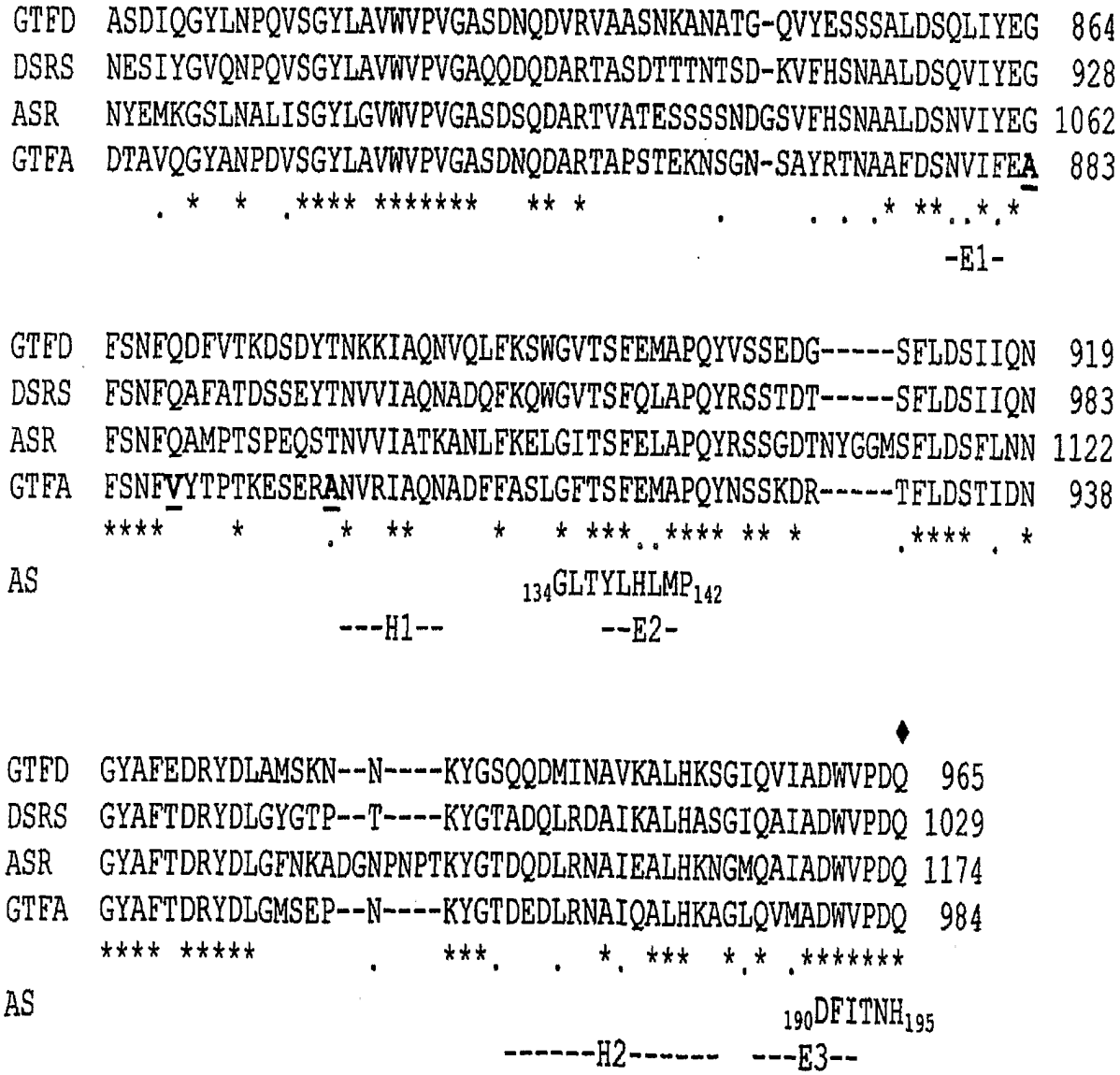
```

Fig.4.3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ASR NYEMKGSLNALISGYLGVWVPVGASDSQDARTVATESSSSNDGSVFHSNAALDSNVIYEG 1062
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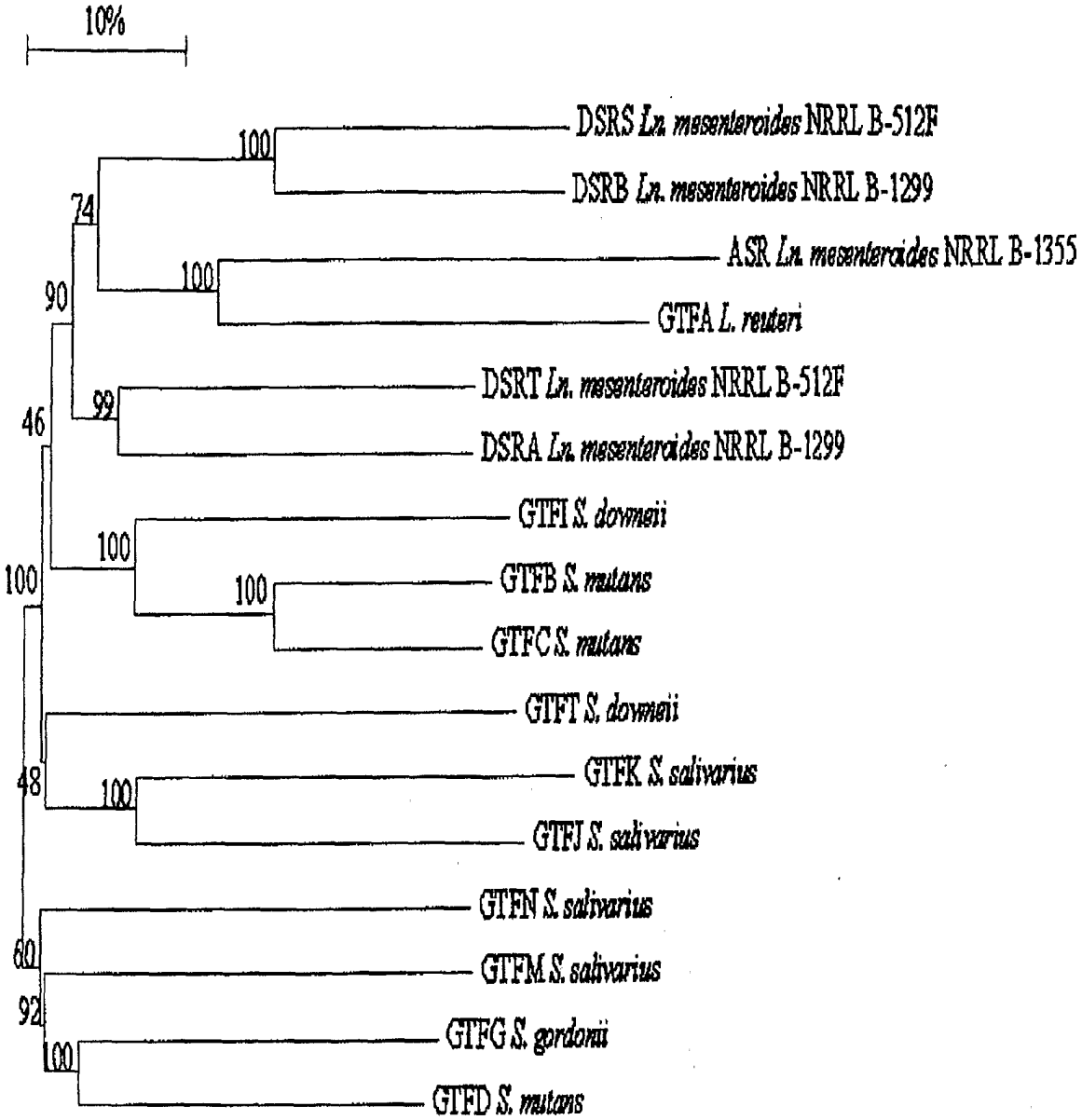
Fig.5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DSRA Ln. mesenteroides NRRL B-1299
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GTFB S. mutans
GTFC S. mutans
GTFT S. downeii
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GTFJ S. salivarius
GTFN S. salivarius
GTFM S. salivarius
GTFG S. gordonii
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Fig.6a

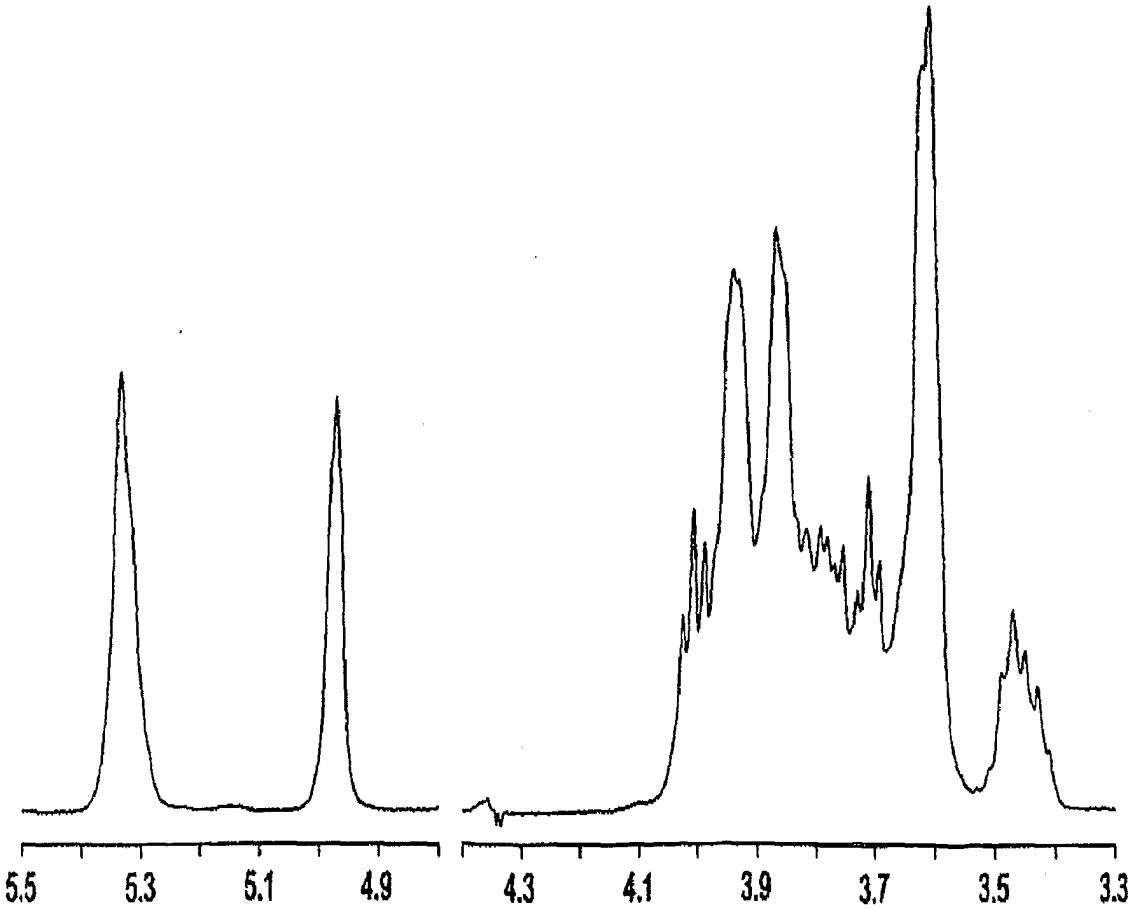
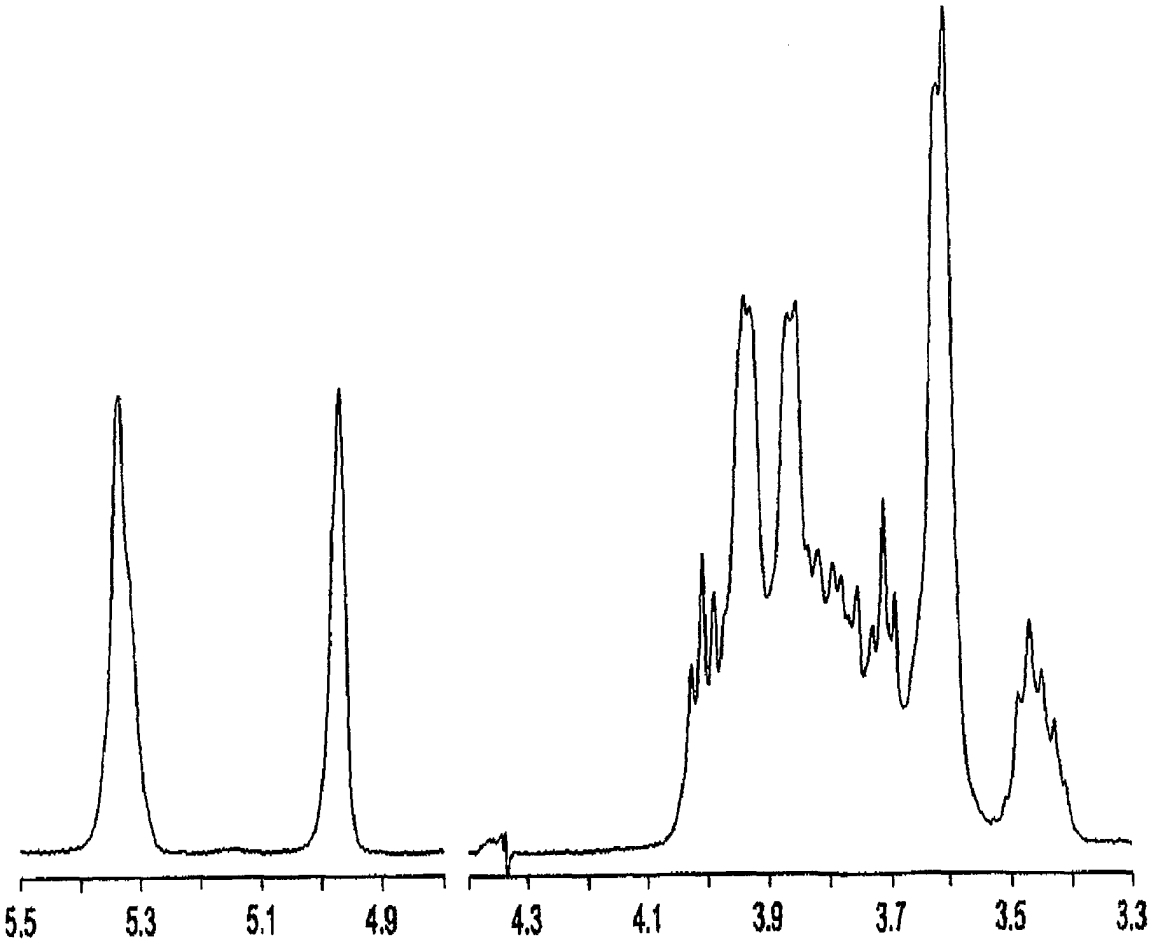


Fig. 6b



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Ala Thr Val Ala Val Ser Thr Ala Leu Leu Tyr Gly Gly Val Ala His
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gct gac caa caa gtt cag caa gct tcc acg act caa gac caa act tct   319
Ala Asp Gln Gln Val Gln Gln Ala Ser Thr Thr Gln Asp Gln Thr Ser
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acc gta aat aat gat act gat aaa aca gta gct tta gat act aat act   367
Thr Val Asn Asn Asp Thr Asp Lys Thr Val Ala Leu Asp Thr Asn Thr
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gac cag tca gct caa aca act gat aaa aaa caa gta gta tca aat act   415
Asp Gln Ser Ala Gln Thr Thr Asp Lys Lys Gln Val Val Ser Asn Thr
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aac caa agc aaa act gat gac act tca aca gct gat aag aat tct act   463
Asn Gln Ser Lys Thr Asp Asp Thr Ser Thr Ala Asp Lys Asn Ser Thr
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Ser Thr Pro Val Ser Val Leu Pro Ser Asn Asn Thr Glu Lys Gln Ala
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aaa aat tat aat gag caa gac aaa gga aac tat ggg aat att gat act   559
Lys Asn Tyr Asn Glu Gln Asp Lys Gly Asn Tyr Gly Asn Ile Asp Thr
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gct tac ttt agc aat aat caa ttg cat gtt tca gga tgg aat gca acg   607
Ala Tyr Phe Ser Asn Asn Gln Leu His Val Ser Gly Trp Asn Ala Thr
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aca acc aat aat gaa tta ggt cgc act gat gta aca aac aat gtt gca   703
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cgc cca gac gtt aag aat gtt cat aat gtt tat aac gct gat aat tct   751
Arg Pro Asp Val Lys Asn Val His Asn Val Tyr Asn Ala Asp Asn Ser
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cgg gat tca att gaa att gtt agt cga tac agt gga aac ggt aaa tct   847
Arg Asp Ser Ile Glu Ile Val Ser Arg Tyr Ser Gly Asn Gly Lys Ser
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230                 235                 240                 245

tat ctt gat aca ttt gaa gtg aaa aat ggc gaa tta cat gca acc gga   943
Tyr Leu Asp Thr Phe Glu Val Lys Asn Gly Glu Leu His Ala Thr Gly
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tgg aat gct act aat agt gcg att aac tat aat cac cat ttt gtg att   991
Trp Asn Ala Thr Asn Ser Ala Ile Asn Tyr Asn His His Phe Val Ile
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Leu Phe Asp Gln Thr Asn Gly Lys Glu Val Ala Arg Gln Glu Val Arg
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gaa ggt caa tca cgc cca gat gtt gct aag gta tat cca caa gta gtt   1087
Glu Gly Gln Ser Arg Pro Asp Val Ala Lys Val Tyr Pro Gln Val Val
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310                 315                 320                 325

gat tat act cac cag tac caa gtt ctt agt cgt tac agc aat tct gat   1183
Asp Tyr Thr His Gln Tyr Gln Val Leu Ser Arg Tyr Ser Asn Ser Asp
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gct cct gct aat caa agt aac cag ggt tat cta gac tca ttt gat att   1279
Ala Pro Ala Asn Gln Ser Asn Gln Gly Tyr Leu Asp Ser Phe Asp Ile
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agt aaa aat ggt gaa gta aca gta act gga tgg aac gct act gac ttg   1327
Ser Lys Asn Gly Glu Val Thr Val Thr Gly Trp Asn Ala Thr Asp Leu
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ggc aaa caa gtt gca tct gct aaa gct gat tta att tca cgt cca gat   1423
Gly Lys Gln Val Ala Ser Ala Lys Ala Asp Leu Ile Ser Arg Pro Asp
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gtt gct aaa gct tat cca aca gta aaa aca gct aca aat tct ggc ttc   1471
Val Ala Lys Ala Tyr Pro Thr Val Lys Thr Ala Thr Asn Ser Gly Phe
            425                 430                 435

aag gta aca ttt aag gtt aat aac tta caa ccg ggt cac caa tac agc   1519
Lys Val Thr Phe Lys Val Asn Asn Leu Gln Pro Gly His Gln Tyr Ser
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gtt gta agt cgt ttc tct gcc gat gaa aat ggt aat ggt aat gat aag   1567
Val Val Ser Arg Phe Ser Ala Asp Glu Asn Gly Asn Gly Asn Asp Lys
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cgc cat aca gat tac tgg ttt agt cca gta ata tta aac cag act gct   1615
Arg His Thr Asp Tyr Trp Phe Ser Pro Val Ile Leu Asn Gln Thr Ala
470                 475                 480                 485

tca aac att gat act att aca atg aca tct aat ggt tta cat att gca   1663
Ser Asn Ile Asp Thr Ile Thr Met Thr Ser Asn Gly Leu His Ile Ala
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ggt tgg atg gca agt gat aac tca att aat gaa aca act cca tac gct   1711
Gly Trp Met Ala Ser Asp Asn Ser Ile Asn Glu Thr Thr Pro Tyr Ala
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att atc ctc aat aat gga aaa gaa gtt act cgt caa aag atg agc tta   1759
Ile Ile Leu Asn Asn Gly Lys Glu Val Thr Arg Gln Lys Met Ser Leu
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acc gcc cgt cca gat gta gca gca gta tat cct tca ctt tat aat agt   1807
Thr Ala Arg Pro Asp Val Ala Ala Val Tyr Pro Ser Leu Tyr Asn Ser
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gct gtt agt ggt ttt gac act act att aaa ttg act aat gat caa tat   1855
Ala Val Ser Gly Phe Asp Thr Thr Ile Lys Leu Thr Asn Asp Gln Tyr
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caa gcg ctt aat ggc caa tta caa gta ttg tta cgt ttt tct aaa gct   1903
Gln Ala Leu Asn Gly Gln Leu Gln Val Leu Leu Arg Phe Ser Lys Ala
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Ala Asp Gly Asn Pro Ser Gly Asp Asn Thr Val Thr Asp Gln Phe Ser
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aaa aat tat gca act act ggt gga aac ttt gac tat gta aaa gta aat   1999
Lys Asn Tyr Ala Thr Thr Gly Gly Asn Phe Asp Tyr Val Lys Val Asn
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ggt aat caa gtt gaa ttt agt ggt tgg cac gca act aac caa tca aat   2047
Gly Asn Gln Val Glu Phe Ser Gly Trp His Ala Thr Asn Gln Ser Asn
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gat aaa gat tca caa tgg att att gtt tta gtt aat ggt aag gaa gta   2095
Asp Lys Asp Ser Gln Trp Ile Ile Val Leu Val Asn Gly Lys Glu Val
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aag cgt caa tta gtt aat gat act aaa gag gga gct gct ggc ttc aac   2143
Lys Arg Gln Leu Val Asn Asp Thr Lys Glu Gly Ala Ala Gly Phe Asn
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cga aac gat gtc tac aaa gta aat cca gct att gaa aac agt tct atg   2191
Arg Asn Asp Val Tyr Lys Val Asn Pro Ala Ile Glu Asn Ser Ser Met
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tct gga ttc caa ggc att att act tta cct gtg aca gtt aaa aac gaa   2239
Ser Gly Phe Gln Gly Ile Ile Thr Leu Pro Val Thr Val Lys Asn Glu
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aat gtc caa ctt gtt cat cgg ttt agt aac gat gtg aag act ggt gaa   2287
Asn Val Gln Leu Val His Arg Phe Ser Asn Asp Val Lys Thr Gly Glu
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ggt aac tat gtt gat ttc tgg tca gag cta atg cct gtt aag gat agc   2335
Gly Asn Tyr Val Asp Phe Trp Ser Glu Leu Met Pro Val Lys Asp Ser
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ttc caa aag ggg aat ggc cca ctt aag caa ttt ggc tta caa act att   2383
Phe Gln Lys Gly Asn Gly Pro Leu Lys Gln Phe Gly Leu Gln Thr Ile
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aac ggt caa caa tat tat att gac cca aca act ggt caa cca cgt aag   2431
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825 830 835

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855 860 865

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870 875 880 885

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890 895 900

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Ile Ser Glu Thr Gly Asn Thr Asp Trp Leu Arg Thr Leu Met His Asp
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935 940 945

gat tta acg cct tat gct aac tct gat tat cgc tta ctt ggt cgg atg 3055
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950 955 960 965

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gat caa gct aat ttt gat tct gta cgg gta gat gca ccg gat aat att   3247
Asp Gln Ala Asn Phe Asp Ser Val Arg Val Asp Ala Pro Asp Asn Ile
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Asp Ala Asp Leu Met Asn Ile Ala Gln Asp Tyr Phe Asn Ala Ala Tyr
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ggt atg gac tca gat gct gtc tca aat aag cat att aat att ctt gaa   3343
Gly Met Asp Ser Asp Ala Val Ser Asn Lys His Ile Asn Ile Leu Glu
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caa ttg aca atg gat gat act att aag aat tcc ctg aat cat ggg ctt   3439
Gln Leu Thr Met Asp Asp Thr Ile Lys Asn Ser Leu Asn His Gly Leu
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tca gat gca act aat cgt tgg gga tta gat gca att gtt cat cag tca   3487
Ser Asp Ala Thr Asn Arg Trp Gly Leu Asp Ala Ile Val His Gln Ser
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Thr Val Lys Lys Tyr Asn Leu Tyr Asn Ile Pro Ala Ser Tyr Ala Ile
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ctt tta act aac aag gat aca att cca cgt gta tac tat ggt gac ttg   3775
Leu Leu Thr Asn Lys Asp Thr Ile Pro Arg Val Tyr Tyr Gly Asp Leu
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tat act gat ggt ggc caa tac atg gaa cat caa aca cgt tac tat gat   3823
Tyr Thr Asp Gly Gly Gln Tyr Met Glu His Gln Thr Arg Tyr Tyr Asp
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act tta acg aac ctg ctt aaa tca cga gtt aag tat gtt gcc ggt ggc   3871
Thr Leu Thr Asn Leu Leu Lys Ser Arg Val Lys Tyr Val Ala Gly Gly
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Gln Ser Met Gln Thr Met Ser Val Gly Gly Asn Asn Asn Ile Leu Thr
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agt gtt cgt tat ggt aaa ggt gcg atg aca gct act gat act ggt act   3967
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gat gaa acc aga aca caa ggt att ggg gtt gtt gta agt aat acg cca   4015
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Asn Leu Lys Leu Gly Val Asn Asp Lys Val Val Leu His Met Gly Ala
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gcg cac aag aac caa caa tat cgg gca gcc gtg ttg acg aca act gat   4111
Ala His Lys Asn Gln Gln Tyr Arg Ala Ala Val Leu Thr Thr Thr Asp
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Gly Val Ile Asn Tyr Thr Ser Asp Gln Gly Ala Pro Val Ala Met Thr
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gac gag aac ggt gat cta tac tta tct agt cat aac cta gtt gtt aat   4207
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Val Ser Gly Tyr Leu Ala Val Trp Val Pro Val Gly Ala Ser Asp Asn
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caa gat gct cga act gct cca tct act gaa aag aat agt ggt aac tct   4351
Gln Asp Ala Arg Thr Ala Pro Ser Thr Glu Lys Asn Ser Gly Asn Ser
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gca tac aga aca aat gct gct ttt gat tca aat gtt att ttt gaa gcc   4399
Ala Tyr Arg Thr Asn Ala Ala Phe Asp Ser Asn Val Ile Phe Glu Ala
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Phe Ser Asn Phe Val Tyr Thr Pro Thr Lys Glu Ser Glu Arg Ala Asn
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gtt cga att gcc caa aat gct gat ttc ttt gct tca tta ggt ttt act   4495
Val Arg Ile Ala Gln Asn Ala Asp Phe Phe Ala Ser Leu Gly Phe Thr
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Ser Phe Glu Met Ala Pro Gln Tyr Asn Ser Ser Lys Asp Arg Thr Phe
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Leu Asp Ser Thr Ile Asp Asn Gly Tyr Ala Phe Thr Asp Arg Tyr Asp
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Leu Gly Met Ser Glu Pro Asn Lys Tyr Gly Thr Asp Glu Asp Leu Arg
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aat gcc att caa gcg ctc cat aaa gct ggc tta caa gta atg gcg gat   4687
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Trp Val Pro Asp Gln Ile Tyr Asn Leu Pro Gly Lys Glu Val Ala Thr
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gtc act cga gta gat gat cgt ggt aat gta tgg aaa gat gct atc att   4783
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aag aag tat ggt gga gca ttc ctc gat aag tta caa aaa ctt tat cct   4879
Lys Lys Tyr Gly Gly Ala Phe Leu Asp Lys Leu Gln Lys Leu Tyr Pro
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gaa atc ttc aca aag aag caa gtt tca act ggt gtt gct att gat cct   4927
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Ser Gln Lys Ile Thr Glu Trp Ser Ala Lys Tyr Phe Asn Gly Thr Asn
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att ctc cat cgt ggt tct ggt tat gta cta aaa gct gat ggt ggt caa   5023
Ile Leu His Arg Gly Ser Gly Tyr Val Leu Lys Ala Asp Gly Gly Gln
               1610                1615                1620

tac tac aac tta ggt act act aca aag caa ttc ttg cca att caa tta   5071
Tyr Tyr Asn Leu Gly Thr Thr Thr Lys Gln Phe Leu Pro Ile Gln Leu
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act ggt gaa aag aaa caa gga aat gaa ggc ttt gtt aag ggt aat gat   5119
Thr Gly Glu Lys Lys Gln Gly Asn Glu Gly Phe Val Lys Gly Asn Asp
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Gly Asn Tyr Tyr Phe Tyr Asp Leu Ala Gly Asn Met Val Lys Asn Thr
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ttt att gaa gat agt gtt ggc aac tgg tac ttc ttt gac caa gat ggt   5215
Phe Ile Glu Asp Ser Val Gly Asn Trp Tyr Phe Phe Asp Gln Asp Gly
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aag atg gtt gaa aat aaa cat ttc gtt gat gtt gat tct tat ggt gaa   5263
Lys Met Val Glu Asn Lys His Phe Val Asp Val Asp Ser Tyr Gly Glu
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aaa ggt act tac ttc ttc ttg aag aat ggt gta tca ttc cgt ggg gga   5311
Lys Gly Thr Tyr Phe Phe Leu Lys Asn Gly Val Ser Phe Arg Gly Gly
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tta gtg caa act gac aat ggt act tat tac ttt gat aat tat gga aag   5359
Leu Val Gln Thr Asp Asn Gly Thr Tyr Tyr Phe Asp Asn Tyr Gly Lys
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Tyr Pro Thr Ser Thr Asp Val Gly Lys Met Leu Asp Gln Asn Lys Leu
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gttgttgtta tatcagtgga tttaagcaac atgaattaat tgaagatgac ggcaatgatt 5736

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Gln Asp Gln Thr Ser Thr Val Asn Asn Asp Thr Asp Lys Thr Val Ala

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Val Val Ser Asn Thr Asn Gln Ser Lys Thr Asp Asp Thr Ser Thr Ala
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Asp Lys Asn Ser Thr Ser Thr Pro Val Ser Val Leu Pro Ser Asn Asn
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Gly Trp Asn Ala Thr Asn Ala Ser Gln Gly Thr Asn Ser Arg Gln Ile
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Ile Val Arg Asp Ile Thr Thr Asn Asn Glu Leu Gly Arg Thr Asp Val
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Thr Asn Asn Val Ala Arg Pro Asp Val Lys Asn Val His Asn Val Tyr
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Asn Ala Asp Asn Ser Gly Phe Asp Val Asn Val Asn Ile Asp Phe Ser
        195                 200                 205

Lys Met Lys Asp Tyr Arg Asp Ser Ile Glu Ile Val Ser Arg Tyr Ser
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Gly Asn Gly Lys Ser Val Asp Trp Trp Ser Gln Pro Ile Thr Phe Asp
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His His Phe Val Ile Leu Phe Asp Gln Thr Asn Gly Lys Glu Val Ala
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Tyr Pro Gln Val Val Gly Ala Ala Asn Ser Gly Phe Asn Val Thr Phe
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Asp Ser Phe Asp Ile Ser Lys Asn Gly Glu Val Thr Val Thr Gly Trp
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Asn Ala Thr Asp Leu Ser Glu Leu Gln Asn Asn His Tyr Val Ile Leu
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Phe Asp Gln Thr Ala Gly Lys Gln Val Ala Ser Ala Lys Ala Asp Leu
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Gly His Gln Tyr Ser Val Val Ser Arg Phe Ser Ala Asp Glu Asn Gly
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Asn Gly Asn Asp Lys Arg His Thr Asp Tyr Trp Phe Ser Pro Val Ile
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645 650 655

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Thr Val Lys Asn Glu Asn Val Gln Leu Val His Arg Phe Ser Asn Asp
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Pro Val Lys Asp Ser Phe Gln Lys Gly Asn Gly Pro Leu Lys Gln Phe
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Gly Leu Gln Thr Ile Asn Gly Gln Gln Tyr Tyr Ile Asp Pro Thr Thr
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Lys Gln His Gly Asn Leu Leu Pro Ser Ala Leu Pro Phe Phe Asn Ala
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995 1000 1005

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INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/NL 01/00393

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/54 C12N9/10 C12N1/21 C12P19/18 C08B37/00
A23C9/123 //(C12N9/10,C12R1:225)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P C08B A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CHEM ABS Data, BIOSIS, EPO-Internal, WPI Data, PAJ, MEDLINE, STRAND, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	VAN GEEL-SCHUTTEN, G. H. ET AL: "Exopolysaccharide production by Lactobacillus reuteri, involving sucrase type of enzymes" MEDED. - FAC. LANDBOUWKD. TOEGEPASTE BIOL. WET. (UNIV. GENT), vol. 65, no. 3a, 2000, pages 197-201, XP000971140 the whole document --- -/--	1-16

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 2001	10/09/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lejeune, R

INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/NL 01/00393

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	VAN GEEL-SCHUTTEN G H ET AL: "Biochemical and structural characterization of the glucan and fructan exopolysaccharides synthesized by the Lactobacillus reuteri wild-type strain and by mutant strains." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 65, no. 7, July 1999 (1999-07), pages 3008-3014, XP002154732 ISSN: 0099-2240 cited in the application	15,16
Y	abstract page 3010, left-hand column, last paragraph -right-hand column page 3013, left-hand column	1-14
Y	MONCHOIS VINCENT ET AL: "Cloning and sequencing of a gene coding for a novel dextransucrase from Leuconostoc mesenteroides NRRL B-1299 synthesizing only alpha(1-6) and alpha(1-3) linkages." GENE (AMSTERDAM), vol. 182, no. 1-2, 1996, pages 23-32, XP004071926 ISSN: 0378-1119 abstract figure 1; table 1	1-14
Y	SIMPSON CHRISTINE L ET AL: "Streptococcus salivarius ATCC 25975 possesses at least two genes coding for primer-independent glucosyltransferases." INFECTION AND IMMUNITY, vol. 63, no. 2, 1995, pages 609-621, XP002154733 ISSN: 0019-9567 the whole document	1-14
X	DATABASE EMBL 'Online! Accession U12643, 16 November 1995 (1995-11-16) VICKERMAN M M ET AL: "Streptococcus gordonii glucosyltransferase (gtfG), Dsg (dsg), RggD (rggD), and YlbN-like hypothetical protein genes, complete cds; and hypothetical protein gene, partial cds." XP002176190 abstract	1,3,9-11